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ALKALINE PHOSPHATASE ACTIVITY IN NORMAL AND ABNORMAL HUMAN BLOOD AND BONE MARROW CELLS

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IN 1907 Winkler¹ demonstrated a staining technique for the microtechnical visualization of the enzyme oxydase in blood films. The demonstration of this and a similar enzyme, peroxydase, has since become a standard technique for the identification of cells of the myeloid series. The oxydase stain apparently represents the first successful method for the histochemical demonstration of enzymatic activity. The microtechnical visualization of alkaline phosphatase activity by Takamatsu² and Gomori³ suggested that a study be made of alkaline phosphatase in films of human blood and bone marrow under normal and pathologic conditions.

METHOD

The technique used was essentially Gomori's method as modified by Kabat and Furth⁴ with small alterations.⁵ Dried blood or bone marrow films were covered for a few seconds, without further fixation, by a solution containing 0.25 Gm. celloidin in 50 c.c. absolute alcohol and 50 c.c. ether. They were then dipped into 95 per cent alcohol, washed in distilled water, and incubated for from ten to fifteen hours at 37° C. in a solution made up freshly each time and containing 10 c.c. of a 3.2 per cent sodium glycerophosphate 5 H₂O,⁶ 10 c.c. of a 10 per cent barbital sodium (sodium diethyl barbiturate) solution, 10 c.c. of m/10 magnesium sulfate, 15 c.c. of a 2 per cent calcium nitrate solution, and 55 c.c. of distilled water. It is advisable to keep the glycerophosphate and sodium barbiturate stock solutions in the refrigerator. The slides were then washed in water containing a few cubic centimeters of the calcium nitrate solution, transferred into a 2 per cent cobalt nitrate solution for five minutes, and, after thorough washing in tap water, brought into a Coplin jar containing a few drops of ammonium sulfide. The tissue phosphatase liberates inorganic phosphate from the substrate, which is precipitated by calcium ions present in the incubating solution; the inorganic phosphate combines with the cobalt nitrate to form cobalt phosphate. The cobalt phosphate is then transformed into black cobalt sulfide.

Preformed calcium present in tissue sections also leads to black deposits. It was necessary, therefore, to incubate sections in a mixture without glycerophosphate. However, repeated controls of this kind did not demonstrate any nonspecific staining in blood films. Whenever possible, three slides were used.

¹From the laboratories of the Elizabeth A. Horton Memorial Hospital, Middletown, N. Y., and the Beth Israel Hospital, Passaic, N. J.

²Received for publication, Aug. 8, 1945.

³Fifty-two per cent alpha, Eastman Kodak Company, Rochester, N. Y.

One was studied without counterstain; one was counterstained for several seconds in a 0.5 per cent aqueous solution of basic fuchsin; and another was counterstained with Harris hematoxylin for one minute and thirty seconds with a 0.5 per cent alcohol solution of eosin. It was necessary to study unstained slides, since any counterstain will cover small amounts of dark deposits indicative of a weak phosphatase activity. Unless mounted with elarite or a similar medium, the stain will fade in a short time.

In contrast to the oxydase reaction, the phosphatase reaction in blood films could be demonstrated even in one-year-old slides in cells which contained large amounts of phosphatase (that is, leucocytes in infections). In the presence of only small amounts of phosphatase, the enzyme was no longer demonstrable after several weeks. It is advisable, therefore, to use fresh preparations.

RESULTS

Normal Subjects.—The blood of twenty-eight subjects was examined. All had normal red blood cell counts as well as normal amounts of hemoglobin. Red blood cells, lymphocytes, monocytes, and eosinophilic leucocytes did not show

TABLE I

SUB- JECT	SEX	W.B.C./C.MM.	NEUTROPHILE LEUCOCYTES			EOSINO- PHILES (PER CENT)	LYMPHO- CYTES (PER CENT)	MONO- CYTES (PER CENT)	PHOSPH. POSITIV NEUTROPI	
			POLY- MORPHO- NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.MM.				PER CENT	NO./
1	F	8,000	65	—	5,200	4	26	5	72	3,
2	F	6,500	56	3	3,835	—	37	5	82	3,
3	M	9,300	56	—	5,208	2	41	1	24	1,
4	M	6,100	70	6	4,636	—	22	2	91	4,
5	M	9,100	70	2	6,552	—	26	2	20	1,
6	F	8,000	64	4	5,440	1	38	3	48	2,
7	F	7,500	66	4	5,250	1	28	1	38	1,
8	F	8,700	72	2	6,438	4	21	1	28	1,
9	F	7,700	74	4	6,006	1	20	1	25	1,
10	F	9,000	54	3	5,130	—	39	4	65	3,
11	F	6,000	64	7	4,260	—	27	2	76	3,
12	M	5,700	78	3	4,617	—	18	1	30	1,
13	F	7,300	75	6	5,913	4	14	1	24	1,
14	M	7,500	63	2	4,875	1	32	2	22	1,
15a	M	7,500	66	2	5,100	4	24	4	7	—
15b	M	6,500	66	6	4,680	2	16	2	18	—
16	F	7,500	65	—	4,875	2	30	3	23	1,
17	M	5,600	48	—	2,688	2	42	8	72	1,
18	M	9,300	60	9	6,417	7	19	5	30	1,
19	M	9,000	52	4	5,040	3	36	5	20	1,
20	F	7,500	52	—	3,900	2	40	6	78	3,
21a	F	9,800	57	3	5,880	2	35	3	24	1,
21b	F	8,650	72	—	6,228	5	21	2	24	1,
22	F	7,500	66	—	4,950	1	31	2	60	2,
23a	F	10,100	50	2	5,252	2	42	4	54	2,
23b	F	7,000	44	2	3,220	2	50	2	30	—
24	F	6,500	61	4	4,225	1	32	2	50	2,
25	F	7,500	60	2	4,650	1	34	3	24	1,
26	F	9,300	54	2	5,208	2	38	4	35	1,
27	M	7,000	55	—	3,850	4	34	6	30	1,
28	F	7,200	52	—	3,744	4	42	3	46	1,
Average		7,737			4,621				41	1.9

*Since phosphatase activity was found mostly in polymorphonuclear and juvenile neutrophils in counterstained preparations, the percentage and number of positive cells were recorded in this group only.

TABLE II

	TIME	W.B.C./C.M.M.	NEUTROPHILE LEUCOCYTES			EOSINOPHILES (PER CENT)	LYMPHOCYTES (PER CENT)	MONOCYTES (PER CENT)	PHOSPHATASE-POSITIVE NEUTROPHILES	
			POLYMORPHO-NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.				PER CENT	NO./C.M.M.
Subject 1	Fasting									
a	8:30 A.M.	6,500	65	3	4,420	5	26	1	39	1,724
b	11:30 A.M.	8,000	57	2	4,720	6	34	1	35	1,652
c	2:30 P.M.	7,700	61	1	4,774	6	31	1	32	1,528
d	4:30 P.M.	8,300	67	1	5,644	6	25	1	32	1,806
Subject 2	Fasting									
a	8:00 A.M.	7,100	56	4	4,260	3	36	1	36	1,534
b	11:15 A.M.	11,000	43	6	5,390	1	51	1	21	1,132
c	3:00 P.M.	10,000	46	6	5,200	3	43	2	40	2,080
d	5:00 P.M.	9,000	45	4	4,110	1	50	0	44	1,940

any phosphatase activity. Among the neutrophiles, a varying number of cells was found to contain alkaline phosphatase (Table I). The number of positive cells varied considerably on different days in the same individual and even during the same day in the same subject (Table II).

The majority of the neutrophiles of normal individuals revealed only weak phosphatase activity. Usually only small portions of the cytoplasm showed small black deposits; however, in some cells the whole cytoplasm stained black. The reaction in the nuclei varied. Usually the nuclei revealed no, or only slight, activity. Occasional cells showed activity only in the nuclei and none in the cytoplasm.

Bone marrow films from four normal subjects were examined for phosphatase activity. The application of the phosphatase stain to bone marrow preparations, however, was not as satisfactory as to blood smears. Not infrequently uneven staining was observed. Groups of cells showed activity while a similar type of cells, when more isolated in other fields, appeared to be devoid of phosphatase. Occasional nucleated red cells, erythroblasts as well as normoblasts, showed activity in their nuclei. However, the majority of these cells was phosphatase negative. Megakaryocytes were, as a rule, negative. Among the

TABLE III

SUBJECT	SEX	NEUTROPHILE LEUCOCYTES IN BLOOD/C.M.M.	PHOSPHATASE POSITIVE NEUTROPHILES IN BLOOD		MYELOID CELLS IN STERNAL MARROW/C.M.M.	PHOSPHATASE POSITIVE MYELOID CELLS IN STERNAL MARROW		RELATION BETWEEN PHOSPHATASE-POSITIVE MYELOID CELLS IN BLOOD AND STERNAL MARROW
			PER CENT	NO./C.M.M.		PER CENT	NO./C.M.M.	
1	M	4,875	22	1,073	80,000	13	10,400	1:9.7
2	M	4,617	30	1,385	150,000	10	15,000	1:10.8
3	M	5,040	20	1,008	49,000	12	5,950	1:5.5
4	M	6,417	30	1,925	113,000	10	11,300	1:5.8
Average		5,237	25.5	1,348	98,000	11.2	10,645	1:7.9

myeloid elements, only polymorphonuclear, as well as occasional juvenile leucocytes, mostly stab forms, showed moderate activity. In each single case, the number of positive cells in the bone marrow was greater than in the peripheral circulation (Table III).

Patients With Infection.—Blood films from thirty-two patients suffering from different kinds of infections were examined, several repeatedly (Tables IV and V). As with the blood of normal individuals, lymphocytes, monocytes, and eosinophilic leucocytes did not show phosphatase activity. Most of the neutro-



Fig. 1.—Two neutrophilic leucocytes showing strong phosphatase activity in a patient with pneumonia. ($\times 1000$.)



Fig. 2.—A polymorphonuclear leucocyte showing strong phosphatase activity in higher magnification. ($\times 2000$.)

TABLE IV

SUBJECT	SEX	DIAGNOSIS	R.B.C./C.M.M.	Hb. (GM.)	W.B.C./C.M.M.	NEUTROPHILE LEUCOCYTES			MYELOCYTES (PER CENT)	EOSINOPHILES (PER CENT)	LYMPHOCYTES (PER CENT)	MONOCYTES (PER CENT)	PHOSPHATASE-POSITIVE NEUTROPHILES	
						POLYMORPH-NUCLEAR (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.					PER CENT	NO./C.M.M.
1	F	Pneumonia	3.8	10.0	42,000	65	30	39,900	-	-	2	3	97	38,703
2	M	Pneumonia	3.6	9.7	21,200	83	7	19,080	-	-	9	1	88	16,703
3	M	Pneumonia	3.6	9.0	32,000	58	30	28,160	-	1	4	4	81	22,809
4	F	Pneumonia	3.7	10.4	15,900	60	9	10,971	-	-	3	1	64	7,021
5	M	Osteomyelitis	4.0	11.05	22,000	75	11	16,500	-	-	14	-	95	14,475
6	F	Pneumonia	3.1	8.05	60,000	89	4	55,800	12	-	4	1	90	50,220
7	M	Pneumonia	4.9	13.85	41,500	56	30	35,690	-	-	8	6	95	33,905
8	M	Retroperitoneal abscess	3.8	10.4	23,500	78	12	21,150	-	-	5	5	94	19,881
9	M	Skin infection	3.9	10.4	23,900	74	14	25,432	-	1	8	3	92	23,397
10	M	Streptococcal infection	4.0	10.4	25,600	68	13	20,736	-	1	10	8	95	19,699
11	F	Puerperal infection	2.6	8.05	41,000	58	39	39,770	-	-	3	-	98	38,974
12	M	Otitis media	3.7	9.7	18,000	60	26	15,480	-	-	14	-	96	14,861
13	M	Pneumonia	2.9	8.35	21,900	76	12	19,272	-	-	12	-	96	18,501
14	F	Diverticulitis	4.7	12.15	14,000	70	16	12,040	-	-	14	-	93	11,197
15	M	Skin infection	3.6	9.4	24,500	80	16	23,320	-	-	4	-	97	22,620
16a	M	Rheumatic fever	2.8	7.4	29,800	72	25	28,906	-	-	3	-	91	26,304
16b	M	Rheumatic fever	2.9	7.05	20,000	58	37	19,000	-	1	3	1	97	18,430
17	M	Perforated gastric ulcer	4.9	15.0	24,000	76	23	23,760	-	-	1	-	96	22,809
18	M	Otitis media	3.0	8.05	18,400	65	21	15,824	-	2	10	2	92	14,558
19	F	Meningitis	3.9	11.8	20,300	64	18	16,646	-	-	16	2	90	14,981
20	M	Pneumonia	2.8	8.7	10,400	79	3	8,528	-	-	15	3	95	8,101
21	M	Meningitis	4.7	12.45	14,800	72	10	12,136	-	-	16	2	97	11,772
22	M	Skin infection	4.5	12.1	12,000	60	22	9,840	-	-	16	6	90	7,872
23	M	Carbuncle	4.0	11.8	17,000	67	25	15,640	-	-	8	-	93	14,545
24	M	Peritonitis	3.7	11.5	34,600	26	68	32,524	2	-	4	-	90	29,268
25	F	Septicemia diabetes	5.5	15.70	47,700	30	62	43,884	2	-	6	-	99	43,445
Average					26,269			23,461					92	21,736

philes in the circulating blood were strongly positive. They contained very large amounts of phosphatase (Fig. 1). Phosphatase was found in both nuclei and cytoplasm. In many cases the latter contained distinctly more enzyme. In cells of this kind, the nucleus stained lighter than the cytoplasm and stood out against the dark background (Fig. 2). Frequently the whole cells were diffusely blackened without any recognizable detail. Polymorphonuclear and juvenile neutrophils participated in this reaction.

In seven patients (Table V) with marked leucocytosis, the usual large number of intensively positive leucocytes was found in the blood stream at the height of the infection. With the return of the white cell count to normal, there was also a marked decrease in the proportion and number of positive leucocytes. Not only did the number return to normal limits, but the amount of enzymatic activity in the single cell was likewise markedly decreased, approximating the amounts found in the blood cells of normal individuals.

The constant and strong reaction of the leucocytes in the blood stream of patients suffering from different infections suggested that examination be made

TABLE V

SUBJECT	SEX	DIAGNOSIS	DATE	R.B.C./C.M.M.	HB. (GM.)	W.B.C./C.M.M.	NEUTROPHILE LEUCOCYTES			EOSINOPHILES (PER CENT)	LYMPHOCYTES (PER CENT)	MONOCYTES (PER CENT)	PHOSPHATASE-POSITIVE NEUTROPHILES	
							POLYMORPHO NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.				PER CENT	NO./C.M.M.
1	F	Pyelitis	12/15	3.5	9.7	24,000	70	13	19,920	-	15	2	88	17,529
			12/18			7,500	27	25	3,900	-	48	-	74	2,886
2	F	Acute parotitis	12/30	4.5	12.17	24,000	82	12	22,560	-	6	-	91	20,529
			1/2	3.6	10.0	20,300	58	18	15,428	12	18	4	90	13,885
			1/5			13,000	56	18	9,620	12	20	4	65	6,253
			1/12			7,500	44	6	3,750	-	50	2	56	2,100
3	F	Meningitis	12/21	3.6	9.4	22,000	45	18	13,560	-	37	-	96	13,117
			12/24			28,000	58	18	21,280	-	20	4	96	20,429
			1/9			9,500	60	6	6,270	-	30	4	70	4,389
4	M	Pneumonia	2/26	4.2	11.45	46,000	57	30	40,020	-	13	-	96	38,419
			2/27			16,000	68	10	12,480	-	22	-	88	10,982
			3/1			12,000	22	8	3,360	-	68	2	76	2,553
			3/5			9,100	48	4	4,732	12	42	4	80	3,785
			3/7			10,000	30	2	3,200	4	61	3	50	1,600
5	M	Pneumonia	11/27	3.6	10.0	20,000	75	17	18,400	-	8	-	96	17,664
			11/30			20,000	76	15	18,200	-	9	-	95	17,290
			12/4			11,000	66	9	8,250	1	22	3	64	5,230
			12/7			10,000	64	8	7,200	2	24	2	66	4,752
6	M	Pneumonia	2/25	4.0	10.75	38,600	84	12	37,056	-	4	-	94	34,932
			3/1			13,200	40	2	5,604	4	50	4	62	3,474
7	M	Pneumonia and otitis media	12/6	3.1	9.4	21,000	60	23	17,430	1	14	2	94	16,384
			12/9			5,000	44	12	2,800	8	28	8	70	1,960

of purulent exudates. Exudates from different sources were available. In all cases the exudates contained predominantly polymorphonuclear leucocytes. The phosphatase stain revealed a uniform picture. After treatment with ammonium sulfide the slides took a dark color. Under the microscope, nearly all cells were found to contain large amounts of enzyme (Fig. 3). The nuclei frequently showed more activity than the cytoplasm. The disintegrating leucocytes liberated large amounts of enzyme which formed amorphous black deposits.

In five cases bone marrow films were examined. The neutrophilic leucocytes, including polymorphonuclear and juvenile (stab and metamyelocyte) forms showed frequently very much stronger activity than observed in cells from normal bone marrow (Fig. 4). Apparently a somewhat greater number

TABLE VI

SUBJECT	SEX	DIAGNOSIS	NEUTROPHILE LEUCOCYTES IN BLOOD/C.M.M.	PHOSPHATASE POSITIVE NEUTROPHILES IN BLOOD		MYELOID CELLS IN STERNAL MARROW/ C.M.M.	PHOSPHATASE POSITIVE MYELOID CELLS IN STERNAL MARROW		RELATION BE- TWEEN PHOSPHATASE- POSITIVE MYELOID CELLS IN BLOOD AND STERNAL MARROW
				PER CENT	NO./C.M.M.		PER CENT	NO./C.M.M.	
1	M	Meningitis	12,136	97	11,772	282,000	34	95,880	1:7.4
2	M	Pneumonia	8,528	95	8,101	200,000	43	86,000	1:10.2
3	M	Skin infection	9,840	80	7,872	56,000	40	22,400	1:2.8
4	M	Skin infection	25,432	92	23,397	250,000	32	80,000	1:3.4
5	M	Streptococcic in- fection	20,736	95	19,699	220,000	54	118,000	1:6.03
Average			15,335	91.8	14,168	201,600	40.6	78,800	1:5.96

of cells showed faint activity than those seen in counterstained slides; since in unstained preparations more cells, including myelocytes, showed occasional slight activity in their nuclei. Nucleated red blood cells were more often phosphatase positive and very occasionally the nucleus of a megakaryocyte showed faint activity. As observed in normal subjects, the bone marrow contained more positive cells than the peripheral blood (Table VI).

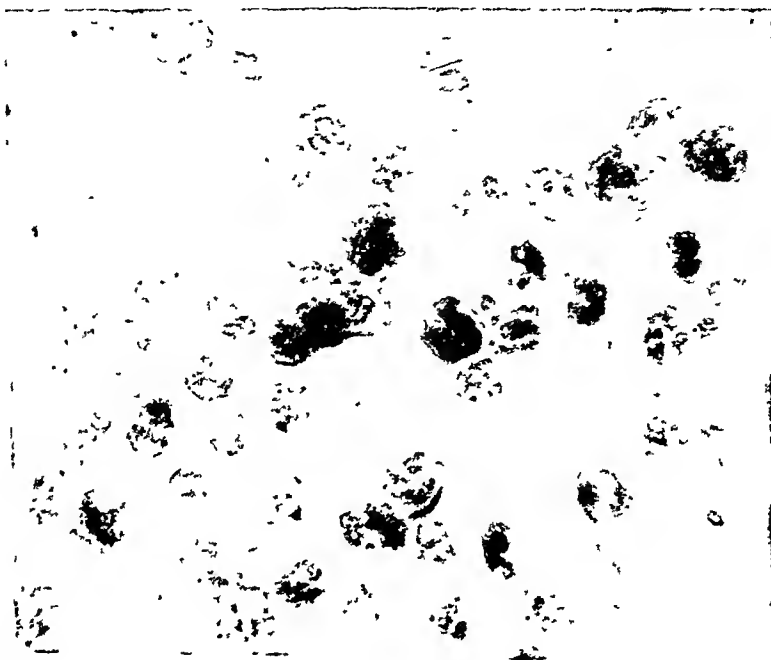


Fig. 3—Polymorphonuclear leucocytes in the spinal fluid of a patient with meningococcus meningitis showing marked phosphatase activity. (X1000.)

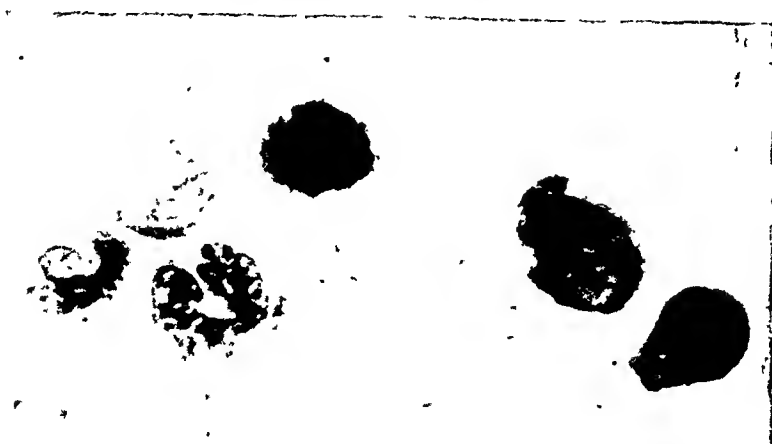


Fig. 4—A group of phosphatase positive myeloid cells in the bone marrow from a patient with pneumonia. (X1000.)

Eosinophilia: Blood films from five patients with marked eosinophilia were available. One patient showed pronounced eosinophilia after pneumonia. In two patients the diagnosis of trichinosis was established and in the remaining two trichinosis was suspected. The total white counts varied between 6,550 and 20,000. The differential count showed between 21 and 42 per cent eosinophilic cells. As seen in normal subjects and in blood films of patients with infections, eosinophilic leucocytes did not show any phosphatase activity. The number of phosphatase positive neutrophils varied between 637 and 4,724 per cubic millimeter.

Infectious Mononucleosis: Four patients with infectious mononucleosis were studied. The white counts varied between 10,000 and 18,400. Between 7 and 36 per cent of the circulating white cells were atypical lymphocytes. Like the normal lymphocytes in the circulating blood, these cells did not show enzymatic activity. In all four patients, neutrophilic leucocytes were partially positive.

Acute Myeloblastic Leucemia: Blood films of fourteen typical instances of acute myeloblastic leucemia were available for study (Table VII). All the patients showed the marked anemia characteristic for this disease. Myeloblasts, promyelocytes, and myelocytes were constantly devoid of any phosphatase activity. Only neutrophils, juvenile as well as polymorphonuclear leucocytes, showed a varying degree of activity. In three of the patients with acute myeloblastic leucemia, no phosphatase positive cells could be found.

TABLE VII

SUBJECT	SEX	R.B.C./C.M.M.	HB. (GM.)	W.B.C./C.M.M.	MYELOBLASTS (PER CENT)	PROMYELOCYTES (PER CENT)	MYELOCYTES (PER CENT)	NEUTROPHILE LEUCOCYTES			EOSINOPHILES (PER CENT)	LYMPHOCYTES (PER CENT)	BASOPHILES (PER CENT)	MONOCYTES (PER CENT)	NUCLEATED RED CELLS FOR 100 W.B.C.	PHOSPHATASE- POSITIVE NEUTROPHILES	
								POLYMORPHO- NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.						PER CENT	NO./C.M.M.
1	M	2.2	5.6	194,000	94	-	-	4	-	7,760	-	3	-	-	-	-	-
2	M	1.8	4.75	5,100	91	1	-	1	-	51	-	7	-	-	1	75	38
3	M	1.6	4.15	5,350	95	-	-	2	-	107	-	3	-	-	1	40	43
4	F	2.3	6.9	40,000	72	-	2	6	8	5,600	6	6	-	-	-	12	672
5	F	1.8	3.8	14,600	50	-	-	6	18	3,504	-	26	-	-	-	8	280
6	F	2.4	6.05	16,000	93	-	-	1	2	480	2	2	-	-	-	90	432
7	F	2.2	6.75	137,000	94	-	-	3	-	4,110	-	3	-	-	-	50	2,055
8	M	1.5	3.8	7,800	64	-	-	-	-	-	-	36	-	-	3	-	-
9	F	2.8	7.05	50,000	33	7	-	5	20	12,500	-	27	7	1	-	40	5,000
10	M	2.77	6.9	380,000	84	3	4	2	1	11,400	-	6	-	-	-	90	10,260
11	M	2.14	5.1	130,000	67	2	3	6	5	14,300	2	14	1	-	-	82	11,726
12	F	2.8	7.05	12,000	82	-	-	1	9	1,200	-	6	-	-	2	-	-
13	M	3.15	7.05	290,000	97	-	-	1	-	2,900	-	2	-	-	-	100	2,900
14	M	2.94	8.05	8,500	44	2	-	34	6	3,400	-	14	-	-	1	26	884

Chronic Myeloid Leucemia: Thirteen typical patients were examined, some of them repeatedly (Table VIII). No phosphatase positive cells were found in six instances, while in one patient a small amount was observed on one occasion and none on another. In five other instances only between 0.5 and 3 per cent phosphatase-positive neutrophils were found. Considering the large number

TABLE VIII

SUBJECT	SEX	R.R.C./C.M.M.	H.B. (G.M.)	W.B.C./C.M.M.	NEUTROPHILIC LEUCOCYTES			MYELOCYTES (PER CENT)	MYELOCYTES POSITIVE (PER CENT)	PROMYELOCYTES (PER CENT)	MYELOBLASTS (PER CENT)	EOSINOPHILS (PER CENT)	BASOPHILS (PER CENT)	MONOCYTES (PER CENT)	LYMPHOCYTES (PER CENT)	NUCLEATED RED CELLS IN 100 W.B.C.	PHOSPHATASE-POSITIVE NEUTROPHILS (PER CENT)
					POLYMORPHO-NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.										
1	F	2.0	4.75	523,000	25	38	329,490	30	4	-	2	4	5	-	2	2	0.5
2	M	3.3	10.0	48,200	7	34	19,762	24	-	7	10	-	5	-	13	1	7
3	F	4.3	11.8	79,200	44	36	63,360	13	-	2	1	-	2	1	1	-	-
4	M	3.8	10.75	92,000	23	30	48,760	34	2	3	4	2	1	-	1	-	-
5	M	3.5	8.7	28,000	45	36	22,680	12	-	-	-	-	-	-	7	4	-
6	M	4.27	10.4	260,000	48	19	174,200	24	-	-	2	3	-	-	4	4	-
7	F	3.8	9.6	40,000	34	42	32,400	5	1	2	2	2	10	-	2	-	15
8	M	3.5	10.0	285,000	52	14	188,100	10	-	16	4	-	3	-	1	1	3
9	M	3.2	8.7	151,800	50	21	107,778	15	4	2	2	3	2	-	1	1	-
10a	M	2.5	5.9	45,000	14	50	28,800	14	1	-	-	2	6	-	13	2	3
10b	M	2.4	6.0	65,900	29	54	54,697	1	-	-	1	-	1	-	14	6	-
11a	F	3.0	8.05	340,000	19	50	234,600	24	2	3	-	-	1	-	1	2	0.6
11b	F	3.8	9.7	365,000	40	47	317,550	5	-	-	-	2	2	-	4	2	3
12	M	4.58	12.4	59,500	38	24	36,890	23	-	2	-	-	11	-	2	-	0.5
13a	F	2.9	7.7	3,000	52	27	2,370	3	-	-	-	2	2	3	11	-	-
13b	F	3.6	9.4	13,500	52	12	8,640	2	-	-	-	2	6	6	20	-	-
13c	F	3.7	9.7	56,000	24	47	39,760	10	-	-	1	-	10	-	8	1	-
13d	F	3.0	8.05	86,200	19	40	50,858	19	-	1	3	2	10	1	5	1	-

of neutrophilic leucocytes in the blood, even these patients showed a marked deficiency in phosphatase-positive cells.

In the blood films of only two patients (Subjects 2 and 7), the proportion of phosphatase-positive leucocytes was within the range found in the blood of normal subjects. In unstained preparations, white cells, including myelocytes, showed occasionally faint activity of their nuclei. These cells showed no activity in the cytoplasm. They were not included in the differential counts, since the application of the counterstain covered this slight activity. The counterstain was, however, essential in order to visualize the phosphatase-negative unstained elements.

Nonleukemic Myelosis: A patient with nonleukemic myelosis appeared to be of special interest. A 56-year-old housewife was known to have had a markedly enlarged spleen for at least eighteen years. The presence of a leucoid blood picture was established for the first time seven years before death. A splenectomy was performed fourteen months before death. Prior to the splenectomy, the white counts had been only moderately elevated with the constant presence of nucleated red blood cells. At the time of death, roentgen ray evidence of sclerosis of the bones was definitely present. The protracted clinical course, the moderately elevated white blood count with a comparatively low proportion of primitive cells, the constant presence of nucleated red blood cells, the chronic splenomegaly, the roentgen ray evidence of osteosclerosis, and the histologic changes in the spleen were fully compatible with the diagnosis of nonleukemic myelosis.⁵⁻⁷

A blood count taken one year after the splenectomy showed 6.1 million red blood cells, 16.45 Gm. hemoglobin, and 47,500 white blood cells, of which 38,950

were neutrophilic polymorphonuclear and juvenile leucocytes. Fifty-seven per cent of the polymorphonuclear and juvenile leucocytes, or 22,301 cells per cubic millimeter, were phosphatase positive. The blood contained at that time 47,500 nucleated red blood cells, all of which were phosphatase negative. About six weeks later, the patient was admitted to the hospital because of the sudden onset of severe pain in chest and abdomen. A blood count now showed 5 million red blood cells, 13.85 Gm. hemoglobin, and 131,000 white blood cells, of which 75 per cent were neutrophilic polymorphonuclear and juvenile leucocytes, or 98,250 cells per cubic millimeter. Fifty-one per cent of these cells, or 50,107 per cubic millimeter, showed phosphatase activity (Fig. 5). In addition the blood contained myelocytes, myeloblasts, and 94,000 nucleated red blood cells per cubic millimeter. None of these cells, with the exception of a few normoblasts, showed enzymatic activity. Only observation of other patients will show whether the phosphatase reaction can be of help in the differentiation of chronic myeloid leucemia from so-called nonleukemic myelosis.

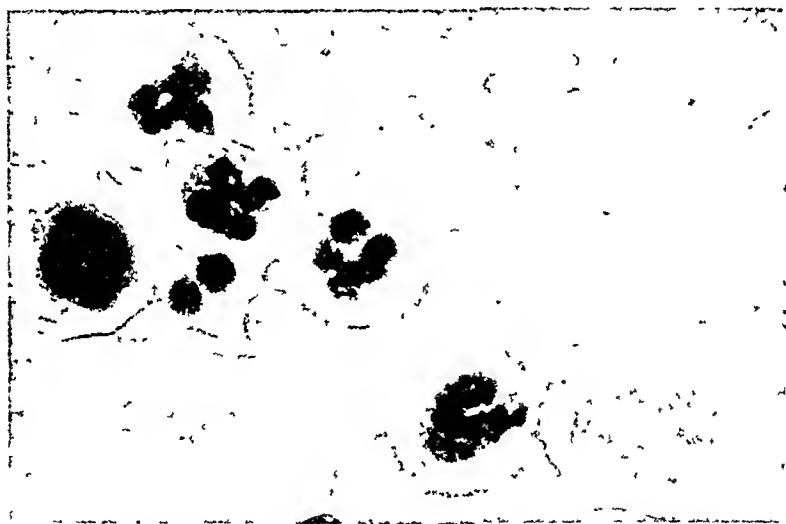


Fig 5.—Chronic nonleukemic myelosis. Several neutrophilic leucocytes showing phosphatase activity. Myelocyte in the right lower corner does not show phosphatase ($\times 1000$.)

Lymphatic Leucemia: Thirteen patients with typical chronic lymphatic leucemia were studied (Table IX). No interference with the enzymatic activity of neutrophilic leucocytes in lymphatic leucemia was found. The lymphatic cells present in the circulatory peripheral blood did not show any activity in twelve instances. Only in Subject 6 did the majority of the leucemic lymphocytes show a moderately strong reaction in the nuclei.

Anemia and Erythroblastosis Fetalis: The blood slides of fifteen patients with anemia and erythroblastosis fetalis were examined (Table X). The nucleated red cells, erythroblasts as well as normoblasts (basophilic, polychromatic, and orthochromatic normoblasts), found in the blood films of several of these

TABLE IX

SUBJECT	SEX	H.B.C./C.M.M.	H.R. (GAL.)	W.B.C./C.M.M.	NEUTROPHILE LEUCOCYTES			LYMPHOCYTES (PER CENT)	LYMPHOBLASTS (PER CENT)	EOSINOPHILES (PER CENT)	BASOPHILES (PER CENT)	MONOCYTES (PER CENT)	NUCLEATED RED CELLS IN 100 W.B.C.	PHOSPHATASE- POSITIVE NEUTROPHILES	
					POLYMORPHO- NUCLEARS (PER CENT)	JUVENILES (PER CENT)	NO./C.M.M.							PER CENT	NO./C.M.M.
1	F	2.3	5.0	204,000	2	-	4,080	94	4	-	-	-	1	20	816
2	F	1.1	3.2	39,000	2	2	1,560	96	-	-	-	-	2	30	468
3	F	2.5	5.6	102,000	1	-	1,020	94	5	-	-	-	1	50	510
4	M	4.9	12.8	54,200	10	5	8,130	81	4	-	-	-	-	75	6,097
5	M	3.9	10.40	45,000	6	-	2,700	94	-	-	-	-	-	20	540
6	M	1.57	5.05	81,000	2	11	10,530	84	2	-	-	1	1	90	9,477
7	M	4.4	10.75	28,300	-	8	2,264	91	-	-	-	1	-	25	566
8	M	2.1	4.75	345,000	1	-	3,450	91	8	-	-	-	-	90	3,105
9	M	4.2	11.8	48,400	2	16	8,712	80	-	2	-	-	-	30	2,614
10	M	1.7	3.55	383,000	1	-	3,830	92	6	1	-	-	1	90	3,447
11a	F	2.34	8.05	187,000	1	-	1,870	94	5	-	-	-	1	90	1,683
11b	F	2.9	9.0	94,000	5	-	4,700	95	-	-	-	-	-	90	4,230
12a	M	4.2	11.8	310,000	1	-	3,100	97	2	-	-	-	-	90	2,790
12b	M	4.0	11.0	280,000	1	-	2,800	96	3	-	-	-	-	90	2,520
13	M	4.2	12.8	328,000	-	18	59,040	82	-	-	-	-	-	84	49,593

patients were, in the majority, devoid of phosphatase activity. This was equally true for the occasionally occurring nucleated cells in cases of leucemia as well as in the previously discussed case of nonleucemic myelosis. However, in one patient with erythroblastosis fetalis most nucleated cells showed phosphatase activity. The positive cells were best seen in preparations in which no counter-stain was used. The activity was always limited to the nucleus.

Bone marrow films from several patients with normocytic and with macrocytic (pernicious) anemia showed activity in varying amounts of white cells and in a fair number of nucleated red cells. The nuclei of megaloblasts as well as of proerythroblasts showed a varying degree of mostly moderate activity.

Different Animals: Blood films from the frog, chicken, rat, guinea pig, rabbit, and dog were examined. The blood cells of the chicken did not contain alkaline phosphatase. Many of the nucleated red cells in the frog showed marked activity. No activity was found in blood films from the dog and mouse. Most of the polymorphonuclear (heterophiles) leucocytes in the blood of guinea pigs, rats, and rabbits showed very strong activity. The amount of activity was comparable to that seen in polymorphonuclear leucocytes in human blood of patients with infectious leucocytosis. The other cells were negative in these species.

Lymph Nodes: In order to study lymphatic elements at the site of their formation, touch preparations from surgically removed tonsils as well as lymph nodes not involved by disease were made. The lymphocytes in these preparations showed a varying behavior. In some, the nuclei were markedly positive, while in the majority only some chromatin particles of the nuclei showed moderate activity. A varying number of lymphocytes was found to be completely devoid of enzymatic activity.

TABLE X

SUBJECT	SEX	DIAGNOSIS	R.B.C./C.M.M.	HB. (GM.)	W.B.C./C.M.M.	NEUTROPHILE LEUCOCYTES				MYELOCYTES (PER CENT)	LYMPHOCYTES (PER CENT)	EOSINOPHILES (PER CENT)	BASOPHILES (PER CENT)	MONOCYTES (PER CENT)	PLASMA CELLS (PER CENT)	NUCLEATED RED CELLS/C.M.M.	PHOSPHATASE-POSITIVE NEUTROPHILES	
						POLYMORPHO-NUCLEARS	(PER CENT)	JUVENILES	(PER CENT)								NO./C.M.M.	
1	F	Pernicious anemia	1.7	5.05	3,500	64	4	-	-	-	32	-	-	-	-	105	30	714
2	M	Pernicious anemia	1.1	4.75	6,000	44	-	-	-	-	56	-	-	-	-	-	44	1,162
3	F	Pernicious anemia	1.4	6.75	8,300	77	-	-	-	-	23	-	-	-	-	83	76	4,857
4	F	Uterine bleeding	2.6	6.05	9,300	75	3	-	-	-	21	1	-	-	-	-	60	4,352
5	F	Bleeding ulcer	2.45	4.75	11,500	80	3	-	-	-	18	-	-	-	-	-	40	3,812
6	M	Secondary anemia and cancer of mandible	2.08	7.05	6,600	50	6	-	-	-	40	-	-	-	-	-	54	1,996
7	F	Chronic nephritis	2.12	5.70	6,800	70	12	-	-	-	16	-	-	-	-	-	20	1,115
8	F	Nutritional anemia	1.72	4.25	15,800	63	-	-	-	-	34	-	3	3	1	-	90	8,959
9	M	Sickle cell anemia	2.0	4.15	9,800	37	19	-	-	-	38	-	3	-	-	-	65	3,567
10	M	Multiple myeloma	1.6	4.15	3,200	37	5	-	-	-	54	-	-	-	-	-	50	672
11	M	Congenital syphilis	2.02	6.4	14,600	10	36	-	-	-	42	4	-	-	4	438	72	4,836
12a	F	Mediterranean anemia	2.0	5.05	29,000	63	3	-	-	-	27	7	-	-	-	91,000	75	14,355
12b	F	Mediterranean anemia	3.0	8.08	42,000	42	8	-	-	-	34	16	-	-	-	73,000	45	9,450
12c	F	Mediterranean anemia	3.0	7.4	26,600	29	9	-	-	-	57	3	1	1	-	5,400	84	8,499
12d	F	Mediterranean anemia	2.0	4.75	25,000	50	10	-	-	-	34	6	-	-	-	100,000	80	12,000
13	F	Erythroblastosis fetalis	2.5	6.4	56,000	58	34	-	-	-	4	4	-	-	-	128,000	88	45,338
14	F	Erythroblastosis fetalis	4.4	15.0	40,000	34	28	-	-	14	22	-	-	-	-	80,000	50	12,400
15	F	Erythroblastosis fetalis (?)	4.2	14.2	12,600	33	33	-	-	-	34	-	-	-	-	6,500	90	7,484

COMMENT

Enzymes in white blood cells, apart from oxydase and peroxydase, have provoked much interest. Proteolytic ferments,¹⁰⁻¹⁶ amylase,^{11, 15-17} maltase,¹⁵ glycolytic enzyme,^{15, 19} lipase,^{11, 15, 16} catalase,²⁰ and nucleotidase²¹ have all been demonstrated. They occur apparently in considerably larger quantities in leucocytes than in lymphocytes.¹⁶ Different enzymes have also been found in leucemic blood cells.^{22, 23}

The fact that the formed elements of the blood contain a phosphatase ester-splitting enzyme was found very soon after phosphatase activity was demonstrated in blood serum. While phosphatase of the red blood cells exerts its optimal activity in an acid medium (ph 4.8 to 6.1),²⁴⁻²⁹ the leucocytes were found to contain a phosphomonoesterase whose optimal activity is around ph 9, like that of the blood serum.^{25-27, 31-33} This phosphatase activity of leucocytes was found to be only weak by Fiessinger and Boyer³³ but very marked by all other investigators in the leucocytes of man and different animals.

Weak activity was reported in the leucocytes of a case of myeloid leucemia by Fiessinger and Boyer. Iwatsuru and Nanjo³⁴ found, however, strong phosphatase activity in the blood of a patient with myeloid leucemia. Normal values were found in the blood of a patient with acute lymphatic leucemia.³⁵ The Japanese investigators³⁶ stated that not only neutrophilic but also eosinophilic leucocytes contain large amounts of alkaline phosphatase. Their results have, however, been doubted because of methodical reasons by Albers.³⁷ Albers found normal phosphatase values in the serum of lymphatic and myeloid leucemias.

In tissue sections strong alkaline phosphatase activity of leucocytes was observed by Gomori in rabbits and guinea pigs³⁸ and by Fell and Danielli in rats.³⁹ Leucocytes are also frequently phosphatase positive in tissue sections from human material. In blood films of the chicken, dog, and mouse, no activity was found, while in guinea pigs, rabbits, and rats most of the polymorphonuclear leucocytes (heterophiles) were strongly positive. Gomori found that in blood films, the leucocytes of guinea pigs were constantly positive, while those of the rat, rabbit, and ground hog were only occasionally the site of phosphatase activity.⁴⁰ The nucleated red cells of frogs showed, in the majority, strong activity. This is in good agreement with the observations of Rapoport and co-workers,⁴¹ who found strong phosphatase activity in frog blood on chemical examination. Contrary to Gomori,³⁸ we found the leucocytes of human blood to reveal phosphatase activity in varying degrees.

By chemical methods phosphatase activity was found in the bone marrow of the mouse.⁴² The findings in tissue sections are somewhat contradictory. Bourne⁴³ found most of the nuclei of bone marrow cells positive, while Takamatsu² and Kabat and Furth,⁴ on the other hand, reported the bone marrow cells to be mostly devoid of phosphatase activity.

In mouse lymph nodes, a moderate amount of alkaline phosphatase activity was found by chemical examination.⁴² This agrees well with the moderate phosphatase activity noticed in touch preparations and paraffin sections from human lymph nodes and tonsils. However, in paraffin sections from human material,

lymphocytes are found to show activity not only at their site of formation, but also in foci of chronic inflammation. The fact that lymphocytes in the circulating blood are negative, but in the tissues frequently positive, has been stressed by Gomori.

The markedly increased phosphatase activity of blood cells in inflammations is interesting. With the subsidence of the inflammatory process in the body, the intensity of the phosphatase reaction in polymorphonuclear leucocytes decreases considerably. In contrast to this phenomenon is the behavior of the circulating leucocytes in chronic myeloid leucemias. In spite of the overproduction of not only immature, but also mature, leucocytes, these cells appear to be deficient in phosphatase activity. The changes of phosphatase activity in leucocytes under abnormal conditions pose the question of the possible significance of alkaline phosphatase activity.

According to Albers,⁴⁴ phosphatase is present in all cells in which carbohydrate metabolism takes place: in plants, in organs of lower and higher animals, as well as in monocellular organisms. Because of the importance of the phosphoric acids for many metabolic processes, phosphatases are one of the most widely distributed enzyme groups. Substrates are carbohydrate-phosphoric acid esters, phosphoproteins, phospholipids, and nucleic acids.

Not too many specific functions, however, have been attributed to the phosphatases. It is probable that they are of great importance for the calcification of living and recently necrosed tissue.³⁸ The wealth of the alkaline phosphatases in the cortex of the kidney, as well as the intestines, has led to the assumption that the enzyme is concerned with the transportation of carbohydrates through the kidney tubules and intestinal mucosa.^{45, 46} Under certain conditions, such as intoxication with uranium nitrate,⁴⁷ hydronephrosis,^{46, 48} choline deficiency,⁶ and scurvy,⁴⁹ decrease in alkaline phosphatase of the kidney has been described. In tissue sections stained for alkaline phosphatase, a very striking activity of the vascular endothelium is seen.^{4, 5, 40, 43}

While the liver, probably the main site for the excretion of alkaline phosphatase, contains only small amounts of this enzyme under normal conditions, very marked increase occurs in protein deficiency, probably due to altered cell metabolism.⁵⁰

Since the introduction of the Warburg technique, the metabolism of leucocytes from exudates as well as from normal and leucemic blood has been repeatedly examined. The previous work has been critically reviewed by Kempner.⁵¹ On the basis of the findings in the literature, as well as of his experiments, Kempner concludes that immature leucocytic cells, both of lymphatic and myeloid origin, have a purely oxidative metabolism without any aerobic lactic acid formation. In contrast, the more mature granulocytes show strong lactic acid formation under aerobic conditions. This is interpreted not as a sign of a malignant quality of these cells, but rather as an expression of their aging and possible damaging influences of the experimental conditions. No significant changes are present between granulocytes from normal and leucemic blood.⁵²

These findings are especially interesting in view of the different behavior of the phosphatase reaction in granulocytes from exudates and of blood during infection, on one hand, and in granulocytes from leucemic blood on the other. The marked increase in alkaline phosphatase activity in the former group indicates apparently the intensification of metabolic processes which are taking place to a lesser degree in granulocytes of normal blood and are markedly depressed or lacking in leucemic blood cells.

SUMMARY AND CONCLUSION

In films of normal human blood, a varying proportion of neutrophilic leucocytes showed alkaline phosphatase activity. The number of positive cells, as well as the intensity of the reaction, increased markedly in infections. The leucocytes in purulent exudates showed strong activity. In bone marrow preparations from normal individuals, as well as from those suffering from infections, a much larger proportion of positive reacting cells was found than in the circulating blood, suggesting that phosphatase activity is already acquired by the leucocytes in the bone marrow. Eosinophiles, as well as lymphocytes, including the abnormal lymphocytes seen in infectious mononucleosis, are devoid of phosphatase activity. In twelve of thirteen patients with lymphatic leucemia, the leucemic cells showed no phosphatase activity. In fourteen patients with acute myeloblastic leucemia, the immature cells were phosphatase negative and there was apparent lack of activity in the remaining granulocytes in two patients. The depression of activity in neutrophiles was more marked in patients with chronic myeloid leucemia, only two of thirteen showing phosphatase activity in these cells comparable to that seen in normal blood films. In one instance of nonleucemic myelosis, many granulocytes gave a strong phosphatase reaction. Circulating nucleated red blood cells showed only occasional activity in their nuclei. These cells were mostly phosphatase negative in bone marrow preparations from normal subjects but were more frequently the site of enzymatic activity in bone marrow of patients suffering from infections as well as from different kinds of anemias.

Among different animals, the neutrophiles (heterophiles) of the guinea pig, rat, and rabbit, and most of the nucleated red cells of the frog showed strong alkaline phosphatase activity, while the blood cells of the chicken, mouse, and dog were negative.

The increased enzymatic activity in neutrophiles under inflammatory stimuli signifies probably increased cell metabolism. While no practical application of the phosphatase reaction for hematologic purposes can so far be recognized, the depression in activity in the granulocytes of chronic myeloid leucemia is of some interest.

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THE EFFECTS OF ADRENALIN AND NEMBUTAL ANESTHESIA ON BLOOD CONSTITUENTS BEFORE AND AFTER SPLENECTOMY

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WITH EXPERIMENTAL COLLABORATION OF J. W. MASON, B.A.

WHILE making observations on members of a party taking a voyage to the Peruvian Andes, Barcroft¹ noted an increase in the concentration of hemoglobin on crossing the equatorial zone and a decline on arriving in colder regions. After some deliberation, he suspected that the spleen might be a hiding place for blood—a hypothesis which Barcroft and associates²⁻⁶ have amply proved by careful experiments on laboratory animals. By the use of x-ray and by methods involving exteriorization of the spleen, they demonstrated that the spleen contracted following hemorrhage, exercise, and death. In addition to other findings, the delayed saturation with carbon monoxide of the hemoglobin in the spleen led to the conclusion that the spleen serves as a blood reservoir. In dogs, they reported that upon contraction, the spleen squeezes into the general circulation an amount of blood equal to about one-fifth of the total blood volume.

In the light of the discovery of this function of the spleen as a blood reservoir, one can clearly understand the basis for the wide ranges in weight of the spleen as given in standard textbooks of anatomy. One book⁷ sets the range between 50 and 400 Gm. while another⁸ puts it between 80 and 300 Gm. In a study of initial and maximal volumes of twenty spleens from unembalmed bodies at post-mortem examination, Wu⁹ found the potential capacity available in the spleen for storage of blood to range from 60 to 400 c.c. Since the spleens were from elderly individuals and since with senescence the expansibility and contractility of the organ diminishes, he stated that the capacity of the "normal" spleen to serve as a blood reservoir should be greater than his findings indicated.

In normal individuals, in patients with splenomegaly, and in splenectomized patients, Yang¹⁰ made a study of the hemoglobin concentration and the red, white, and differential cell counts before and after subcutaneous injection of from 0.5 to 1 c.c. of 1:1000 adrenalin. He observed a marked decrease in the size of the organ in patients with splenomegaly and a definite increase in hemoglobin concentration and in the red and white cell counts during its contraction following adrenalin injection. The increase in red cell counts ranged from 430,000 to 1,100,000; that of hemoglobin, from 4 to 20 per cent; and that of the white cells, from 5,000 to 27,000. In normal individuals the increase in blood constituents was not as marked as in those with enlarged spleens. Splenectomized patients showed no increase in hemoglobin or red cells after adrenalin injection, but the white cells showed increases ranging from 2,000 to 10,000. These increases in blood constituents were attributed to contraction of the spleen

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following administration of adrenalin. In another communication, Yang and Chang¹¹ stated that accompanying the polycythemia and splenic contraction, produced by subcutaneous injection of adrenalin into normal and splenomegalic human subjects, there was no appreciable change in blood volume. Their splenectomized patients showed no significant polycythemia following injection of adrenalin. In studies similar to those of Yang, Benhamou¹² observed that upon administration of adrenalin, moderate but transient increases in the red cell counts and platelets occurred except in the splenectomized patients. The lack of increase in cell counts in the splenectomized patients was attributed to the absence of the spleen. In all patients, however, Benhamou noted a persistent leucocytosis and, consequently, concluded that the contraction of the spleen played no role in the increase of white cells.

In a study on splenomegalic patients and on three individuals after splenectomy, Sehenk¹³ obtained, in every instance, a rise in the red cell count, the white cell count, and in the hemoglobin concentration following subcutaneous administration of from 1 to 2 mg. adrenalin. In his observations on a series of patients with hemolytic jaundice and macrocytic hemolytic anemia, Watson¹⁴ repeatedly noted that subcutaneous injection of adrenalin produced a marked increase in the number of circulating erythrocytes which was usually associated with an obvious shrinkage of the enlarged spleen. At the time of splenectomy for various diseases, Watson and Paine¹⁵ studied the hematocrit, hemoglobin concentration, and the erythrocyte content of venous blood from the spleen before and after injection of adrenalin into the splenic artery. They obtained a marked increase in the erythrocyte content after administration of adrenalin. However, this increase in red cell count was associated with a decrease in mean corpuscular hemoglobin concentration. They attributed the decrease in hemoglobin to a possible intracorpuseular degradation of a fraction of the hemoglobin within the erythrocytes during their sequestration in the spleen. They observed that under the influence of adrenalin the contraction of the spleen was so marked in some cases that it facilitated the delivery of an enlarged spleen through the abdominal incision. In a study of the effect of subcutaneous injection of adrenalin on blood constituents in patients before and after splenectomy, Patek and Daland¹⁶ obtained no significant change in the red cell count, hematocrit or hemoglobin concentrations. In all cases, however, they obtained a leucocytosis which was not any different in patients with spleens than in those after splenectomy. They concluded that the changes observed were not due to contraction of the spleen but to a mechanical alteration in the blood stream. They suggested that the white cells lying along the periphery of blood vessel walls, or pooled in inactive beds, were thrust into the axial stream by active contractions of the blood vessel or by an increased velocity of the axial stream. In a patient having both anemia and splenomegaly, Miller and Rhoads¹⁷ induced and, by the use of x-ray, observed contraction of the spleen by intravenous injection of liver extract and by intramuscular injection of adrenalin, histamine, or eserine. A marked increase in the cellular elements of the blood followed the contraction of the spleen. By means of x-ray they observed that an intramuscular injection of 1 c.c. of adrenalin caused contraction of the spleen from an area

of 109.0 sq. cm. to an area of 48.7 sq. cm. Accompanying this splenic contraction the concentration of hemoglobin in the circulating blood increased 19 per cent and the white blood cells increased 172 per cent.

In a monograph on the physiology of the spleen, Lauda¹⁸ cited evidence indicating that contraction of the spleen is an important factor in the production of an increase in the red cell count after injection of adrenalin and that the erythrocytes from the splenic vein are less resistant to hypotonic saline than those from the splenic artery. Lauda and Haam¹⁹ reported that in dogs under chloralose anesthesia the increase in the red cell content of the blood is brought about by the outpouring of blood from the contracting spleen upon injection of adrenalin. They stated that chloralose anesthesia leads to engorgement of the spleen. In splenectomized animals, they obtained no increase in the red cell content of circulating blood upon administration of adrenalin. They observed that in the rat the blood reservoir function of the spleen is of much smaller magnitude than it is in the dog. Bergenhem and Fahraeus²⁰ suggested that the reservoir function of the spleen is not primarily for keeping a portion of the blood out of circulation as a reserve, but that along with this, a chemical change is produced in lecithin leading to the formation of lysolecithin, which plays an important role in the destruction of red blood cells. Schäfer and Moore²¹ obtained marked contraction of the spleen upon administration of extracts from the adrenals. Lim and Hou²² demonstrated that both the denervated and the innervated halves of the divided spleen in dogs contracted during exercise as a result of the liberation of adrenalin.

By direct transillumination of the mammalian spleen in vivo, MacKenzie and associates²³ observed that adrenalin causes a rapid emptying of the erythrocytes from the splenic pulp. Edmunds and Stone²⁴ obtained an increase in white blood cells, red blood cells, and hemoglobin concentration after injection of adrenalin into dogs in the presence of the spleen as well as after splenectomy. Frey²⁵ reported that the characteristic leucocytosis in intact rabbits, after administration of adrenalin, disappeared after splenectomy. However, in guinea pigs, removal of the spleen had no effect on the response. He reported on a splenectomized patient in whom leucocytosis was obtained after administration of adrenalin. Cruickshank²⁶ observed that in cats the increase in hemoglobin concentration of the fluid expelled from the splenic pulp upon stimulation of the nerves along the splenic artery, amounted to from 20 to 40 per cent above that of normal peripheral blood. He stated that the amount of blood which could be expelled into the general circulation by contraction of the spleen resulting from stimulation of the splanchnic nerves ranged from 2.6 to 5.6 per cent of the total blood volume of the animal. This amount was double the post-mortem weight of the spleen.

Several investigators²⁷⁻³⁰ reported that, upon administration of adrenalin, the intrahepatic vessels are constricted and the circulation of the liver is impeded with a consequent increase in lymph flow and in transudation of fluid. This gave support to the suggestion that, upon injection of adrenalin, the spleen expels chiefly corpuscles into the general circulation, while the plasma which

accompanies the corpuscles is absorbed during its passage through the liver and, consequently, the circulating blood volume is not increased. McLaughlin suggested that the constriction of the smooth muscles in the walls of the hepatic veins increased the filtration pressure and caused a greater production of lymph. Lamson stated that constriction of the hepatic veins with loss of plasma contributes to the increase in red blood cell counts and hemoglobin concentration following administration of adrenalin.

We have frequently observed a striking increase in the size of the spleen at laparotomy performed on dogs anesthetized with nembutal (pentobarbital sodium). Furthermore, it has been reported that during anesthesia with barbiturate compounds the circulating red blood cells are decreased.³¹⁻³⁴ After outlining the spleen with pellets of lead, Hausner, Essex, and Mann³⁵ observed by means of roentgenograms that anesthesia with barbiturates caused marked enlargement of the spleen. They also found a definite reduction in the number of erythrocytes and in the concentration of hemoglobin in the peripheral blood. In their study of the effect of acute hemorrhage in intact and splenectomized dogs under anesthesia with pentobarbital sodium, Carr and Essex³⁶ reported that acute hemorrhage in the splenectomized dog caused a rapid decrease in the concentration of hemoglobin, while in the intact dog acute hemorrhage produced an increase followed by a slow decrease in the concentration of hemoglobin. They obtained practically no decrease in concentration of hemoglobin by anesthetizing splenectomized dogs with pentobarbital sodium.

The purpose of this study was to investigate the effects of adrenalin on the blood constituents in dogs under nembutal anesthesia and without anesthesia before and after splenectomy. We were interested in securing data that might help answer the following questions: (1) Would adrenalin increase the blood cell count and the hemoglobin concentration after surgical removal of the spleen? If so, to what extent? (2) Would adrenalin have any effect on the concentration of total plasma proteins in the presence, as well as in the absence, of the spleen? (3) What is the difference in concentration of the various constituents in blood from the external jugular and from the splenic veins during contraction of the spleen under the influence of adrenalin? (4) Would nembutal anesthesia bring about hemodilution after splenectomy? (5) What effect would nembutal anesthesia have on the concentration of plasma proteins before and after splenectomy?

METHODS

Six adult healthy dogs weighing between 7 and 10 kilograms kept under identical laboratory conditions were used in this study. The hair on the ventral surface of the neck was shaved and blood samples were withdrawn from the external jugular vein and oxalated to prevent coagulation. The red and white cell counts were made by the usual procedure using Hayem solution for the red count and 1 per cent acetic acid for the white count. The concentration of hemoglobin was determined by the use of the Sheard-Sanford³⁷ photometric method. The total plasma proteins were determined by the micro-Kjeldahl method.

Three times a week, control values of the red cell count, white cell count, hemoglobin concentration, and plasma protein concentration were determined on each of the dogs used in this study. After control values were established, similar determinations were made on the same dogs immediately before and toward the end of the second minute after an intravenous injection of 0.5 c.c. of 1:1000 solution of adrenalin hydrochloride. After the effects of adrenalin alone were established, another series of determinations was made on each of the dogs as follows: Control blood samples were withdrawn, after which the animal was given, per kilogram of body weight, 30 mg. of nembutal in physiologic saline, intraperitoneally. Half an hour later, while the animal was under nembutal anesthesia, another sample of blood was withdrawn and was followed by an intravenous injection of 0.5 c.c. of 1:1000 adrenalin hydrochloride; before the end of the second minute after the adrenalin injection, another blood sample was taken. The same series of determinations—those for controls, those after adrenalin alone, and those after nembutal anesthesia and adrenalin—were repeated about one week after splenectomy was performed on each of the dogs used in this investigation. After laparotomy was made under nembutal anesthesia, the outer surface of the spleen was computed before and after the injection of adrenalin and a blood sample was taken from the splenic vein while the spleen was contracting consequent to the adrenalin administration.

The lateral surface of the spleen was determined by laying near its external surface a sterile cloth and cutting it in line with the contour of the spleen. This was done before and after injection of adrenalin. Splenectomy was performed according to the usual standard surgical procedure and under aseptic technique. All six animals had an uneventful recovery.

RESULTS

Changes in Concentration of Hemoglobin in Circulating Blood.—

Before Splenectomy: Blood samples taken from the external jugular vein about 100 seconds after the intravenous injection of 0.5 c.c. of 1:1000 adrenalin hydrochloride solution into intact, unanesthetized dogs before splenectomy showed an average increase in hemoglobin concentration of 1.2 Gm. with a range of from 0.6 to 2.8 Gm. per 100 c.c. of blood. Expressed in percentage, the increase in hemoglobin produced by adrenalin before splenectomy and without anesthesia averaged 9.1 per cent, with a range of from 4.3 to 22.7 per cent over the control values.

The same dose of adrenalin similarly administered into the same intact dogs under nembutal anesthesia produced a greater increase in the concentration of hemoglobin than it did without anesthesia. Expressed in grams, the increase in hemoglobin produced by adrenalin under anesthesia averaged 3.5 Gm., with a range of from 0.9 to 5.0 Gm. per 100 c.c. of blood. Expressed in percentage, the increase in hemoglobin averaged 32.7 per cent, with a range of from 7.0 to 53.8 per cent over the values under anesthesia before adrenalin was administered (Tables I, II, and III).

Nembutal anesthesia alone caused a reduction in the concentration of hemoglobin in circulating blood. Thirty minutes after the onset of nembutal

TABLE I. SHOWING CHANGES IN BLOOD CONSTITUENTS PRODUCED BY ADRENALIN AND NEMIBUTAL ANESTHESIA BEFORE AND AFTER SPLENECTOMY

	BEFORE SPLENECTOMY					AFTER SPLENECTOMY				
	ADRENALIN WITHOUT ANESTHESIA		ADRENALIN WITH NEMIBUTAL ANESTHESIA			ADRENALIN WITHOUT ANESTHESIA		ADRENALIN WITH NEMIBUTAL ANESTHESIA		
	CONTROL	100 SEC. AFTER ADREN.	CONTROL	30 MIN. AFTER ANES.	100 SEC. AFTER ADREN.	CONTROL	100 SEC. AFTER ADREN.	CONTROL	30 MIN. AFTER ANES.	100 SEC. AFTER ADREN.
1										
R.B.C. (millions/mm. ³)	6.66	7.75	6.10	5.60	6.99	5.53	6.22	5.49	4.58	5.28
W.B.C. (thousands/mm. ³)	23.1	21.5	19.4	15.5	21.9	11.1	15.6	52.5	41.1	44.2
Hemoglobin (Gm. %)	13.9	15.5	11.2	11.5	15.4	13.5	13.8	12.4	11.7	12.1
Plasma protein (Gm. %)	5.52	5.62	5.63	5.18	5.61	5.92	6.02	5.89	5.67	5.41
2										
R.B.C. (millions/mm. ³)	5.79	5.94	5.25	4.33	4.38	5.43	5.58	5.24	4.96	5.18
W.B.C. (thousands/mm. ³)	12.1	13.4	8.8	7.9	10.9	12.4	12.3	9.0	7.8	8.3
Hemoglobin (Gm. %)	13.2	13.9	12.1	10.6	15.5	12.1	12.9	12.1	11.7	12.4
Plasma protein (Gm. %)	5.23	5.19	5.38	5.10	5.16	5.93	6.11	5.69	5.31	5.50
3										
R.B.C. (millions/mm. ³)	6.13	6.84	5.87	4.97	7.25	5.72	6.21	5.34	4.93	5.14
W.B.C. (thousands/mm. ³)	11.5	18.9	11.2	9.5	13.6	11.9	15.8	8.6	8.1	9.1
Hemoglobin (Gm. %)	15.5	16.5	14.2	12.1	16.7	14.6	15.6	12.7	12.2	12.9
Plasma protein (Gm. %)	5.00	5.09	5.11	4.78	5.15	4.87	4.99	4.93	4.76	4.90
4										
R.B.C. (millions/mm. ³)	5.91	6.29	5.61	4.19	5.12	5.07	5.29	5.31	4.96	5.11
W.B.C. (thousands/mm. ³)	15.0	17.8	14.1	10.5	16.5	16.7	16.3	18.3	15.5	17.8
Hemoglobin (Gm. %)	13.8	14.4	13.9	10.9	13.0	12.5	12.9	12.1	11.9	12.3
Plasma protein (Gm. %)	5.32	5.38	5.50	4.84	4.97	5.51	5.46	5.80	5.51	5.47
5										
R.B.C. (millions/mm. ³)	6.18	6.98	6.65	5.07	6.22	5.87	5.99	4.68	4.38	4.81
W.B.C. (thousands/mm. ³)	14.0	18.5	14.8	13.1	17.6	29.9	26.2	24.2	20.7	22.2
Hemoglobin (Gm. %)	15.4	16.1	14.9	12.8	13.7	13.1	13.3	11.5	11.2	11.3
Plasma protein (Gm. %)	5.73	5.73	5.99	5.55	5.64	5.77	5.78	5.81	5.55	5.55
6										
R.B.C. (millions/mm. ³)	5.51	6.76	5.44	4.32	5.79	6.20	5.71	5.62	5.09	5.40
W.B.C. (thousands/mm. ³)	14.1	17.1	13.9	11.8	14.9	32.1	29.9	20.0	14.5	17.5
Hemoglobin (Gm. %)	12.3	15.1	11.7	9.3	14.3	13.5	11.0	12.2	11.5	12.5
Plasma protein (Gm. %)	5.53	5.66	5.58	5.06	5.18	5.80	5.85	5.79	5.63	5.61

TABLE II. EFFECT OF NEMBUTAL ANESTHESIA ON CONCENTRATION OF HEMOGLOBIN IN CIRCULATING BLOOD BEFORE AND AFTER SPLENECTOMY

BEFORE SPLENECTOMY					AFTER SPLENECTOMY				
HEMOGLOBIN CONCENTRATION GM./100 C.C.					HEMOGLOBIN CONCENTRATION GM./100 C.C.				
DOG	BEFORE ANES.	30 MIN. AFTER ANES.	DIFFERENCE		BEFORE ANES.	30 MIN. AFTER ANES.	DIFFERENCE		% DIFFERENCE BEFORE AND AFTER SPLENECTOMY
			GM.	%			GM.	%	
1	14.2	11.5	2.7	19.01	12.4	11.7	0.7	5.64	13.37
2	12.1	10.6	1.5	12.4	12.1	11.7	0.4	3.3	9.1
3	14.2	12.3	1.9	13.38	12.7	12.2	0.5	3.93	9.45
4	13.9	10.9	3.0	21.58	12.4	11.9	0.5	4.0	17.58
5	14.9	12.8	2.1	14.09	11.5	11.2	0.3	2.6	11.49
6	11.7	9.3	2.4	20.51	12.2	11.5	0.7	5.74	14.77

TABLE III. EFFECTS OF ADRENALIN WITH AND WITHOUT NEMBUTAL ANESTHESIA ON CONCENTRATION OF HEMOGLOBIN IN CIRCULATING BLOOD BEFORE AND AFTER SPLENECTOMY

% INCREASE PRODUCED BY ADRENALIN WITHOUT ANESTHESIA				% INCREASE PRODUCED BY ADRENALIN DURING ANESTHESIA		
DOG	% BEFORE SPLENECTOMY	% AFTER SPLENECTOMY	% DIFFERENCE	% BEFORE SPLENECTOMY	% AFTER SPLENECTOMY	% DIFFERENCE
1	11.51	2.22	9.29	33.90	3.42	30.48
2	5.30	4.00	1.30	46.20	5.98	40.22
3	6.45	2.74	3.71	35.70	5.73	29.97
4	4.35	3.20	1.15	19.27	3.36	15.91
5	4.55	1.53	3.02	7.03	0.89	6.14
6	22.76	3.71	19.05	53.76	8.70	45.06

anesthesia, blood samples taken from the external jugular vein of intact (not splenectomized) dogs showed a reduction in hemoglobin averaging 2.3 Gm., with a range of from 1.5 to 3.0 Gm. per 100 c.c. of blood as compared with the control values obtained before the administration of nembutal. This reduction in hemoglobin concentration expressed in percentage averaged 16.8 per cent, with a range of from 12.4 to 21.5 per cent, as compared with the control values before anesthesia.

After Splenectomy: After the same dogs were splenectomized, administration of adrenalin (same amount and route as stated previously) without nembutal anesthesia produced an average rise in hemoglobin concentration of 0.4 Gm., with a range of from 0.2 to 0.5 Gm. per 100 c.c. of blood. Expressed in percentage, the increase in hemoglobin concentration averaged 2.9 per cent, with a range of from 1.5 to 4.0 per cent over the control values before adrenalin was administered. Under nembutal anesthesia, the same intravenous dose of adrenalin given to the same splenectomized dogs produced an average increase in hemoglobin concentration of 0.6 Gm., with a range of from 0.1 to 1.0 Gm. per 100 c.c. of blood. Expressed in percentage, the increase in hemoglobin averaged 4.7 per cent, with a range of from 0.9 to 8.7 per cent over the values obtained under anesthesia before adrenalin was given (Tables I, II, and III).

After splenectomy, nembutal anesthesia alone (under the same conditions as stated before removal of the spleen) caused a reduction in the concentration of hemoglobin averaging 0.5 Gm., with a range of from 0.3 to 0.7 Gm. per 100 c.c. of blood. Expressed in percentage, the reduction in concentration of hemo-

globin in splenectomized dogs under the influence of nembutal anesthesia averaged 4.2 per cent, with a range of from 2.6 to 5.7 per cent, as compared with the control values before administration of the anesthetic.

Analysis of the data indicates definitely that nembutal anesthesia caused hemodilution and reduction in the concentration of hemoglobin in intact as well as in splenectomized dogs; however, the hemodilution is greater and the average percentage reduction in the concentration of hemoglobin is four times greater before removal of the spleen than after splenectomy.

Changes in Red Cell Counts in Millions Per Cubic Millimeter.—

Before Splenectomy: The intravenous administration of 0.5 c.c. of 1:1000 adrenalin chloride solution without anesthesia caused an average rise of 0.63 million red cells per cubic millimeter, with a range of from 0.15 to 1.25 million in the total red cell count over that of the control. Nembutal anesthesia caused an average reduction in the total red cell count of 0.98 million per cubic millimeter, with a reduction range of from 0.80 to 1.14 million red cells per cubic millimeter. The same dose of adrenalin as mentioned, given one-half an hour after the onset of nembutal anesthesia, caused an average rise in the red cell count of 1.40 millions per cubic millimeter, with a range in rise of from 0.55 to 2.28 millions per cubic millimeter over the count taken under anesthesia before adrenalin was administered (Table I).

After Splenectomy: After removal of the spleen, intravenous administration of 0.5 c.c. of 1:1000 solution of adrenalin chloride without anesthesia produced a slight increase in the red cell count in five dogs ranging from 0.12 to 0.69 million per cubic millimeter; in the sixth dog there was a slight reduction which is probably an error in the count, because the hemoglobin showed an increase as seen in Table III. After removal of the spleen, nembutal anesthesia still caused a reduction in the red cell count averaging 0.46 million per cubic millimeter, with a reduction range of from 0.28 to 0.91 million red cells per cubic millimeter, as compared with the control red cell count before nembutal was administered. After splenectomy, the intravenous administration of the same dose of adrenalin, one-half an hour after the onset of nembutal anesthesia, still produced a rise in red cell count averaging 0.39 million per cubic millimeter, with a range of from 0.21 to 0.70 million per cubic millimeter, as compared with the count under anesthesia before adrenalin was administered.

Comparison of the changes produced in the red cell counts before and after splenectomy indicates that before removal of the spleen the most significant change in the red cell count is produced by adrenalin when the animal is under anesthesia. In addition to hemodilution, nembutal anesthesia causes relaxation and engorgement of the spleen. This accounts, at least in part, for the difference in increase of the red cell count in the dogs before and after splenectomy under the influence of adrenalin and nembutal anesthesia (Table I).

Changes in White Cell Counts in Thousands Per Cubic Millimeter.—

Before Splenectomy: The intravenous administration of 0.5 c.c. of 1:1000 solution of adrenalin chloride without anesthesia caused an average rise of 3.4 thousand, with a range of from 1.3 to 7.4 thousand in the total white cell count

above the control value. Nembutal anesthesia alone produced a reduction averaging 2.3 thousand, with a range of from 0.9 to 3.9 thousand; as compared with the control count which was made immediately before the administration of the anesthetic. The same dose of adrenalin administered one-half an hour after the onset of nembutal anesthesia produced an average rise of 4.5 thousand, with a range of from 3.0 to 6.4 thousand above the count taken before the administration of adrenalin (Table I).

After Splenectomy: After removal of the spleen, intravenous administration of 0.5 c.c. of 1:1000 solution of adrenalin chloride without anesthesia produced an increase of about 4 thousand in the white cell count in each of two dogs, as compared with the control, while in the other four dogs there was a slight reduction ranging from 0.1 to 3.7 thousand per cubic millimeter. Nembutal anesthesia, after removal of the spleen, caused a reduction of the white cell count averaging 3.8 thousand, with a range of from 0.5 to 9.4 thousand white cells per cubic millimeter below the control values. After splenectomy, the intravenous administration of adrenalin under nembutal anesthesia caused an average increase in the white cell count of 1.6 thousand, with a range of from 0.5 to 3 thousand white cells per cubic millimeter. After splenectomy, the control white cell counts in Dogs 1, 5, and 6 (Table I) showed significant increases over the control counts before removal of the spleen. This is attributable to post-operative reaction.

Changes in Concentration of Plasma Proteins.—The total plasma protein concentration in the unanesthetized dogs was not significantly altered following the administration of adrenalin either before or after splenectomy. The slight variations in plasma protein level as shown in Table I remained within the experimental error of the method. Nembutal anesthesia before splenectomy produced a slight reduction in the concentration of plasma proteins averaging 0.4 Gm. per cent, with a range of from 0.15 to 0.55 Gm. per cent. After splenectomy, anesthesia caused a reduction in plasma protein concentration averaging 0.22 Gm. per cent, with a range of from 0.12 to 0.36 Gm. per cent. Except for the slight dilution of the plasma proteins that was produced by nembutal anesthesia, the other alterations in the plasma proteins under the various experimental conditions are of such a magnitude that they can be considered insignificant (Table I).

Changes in the Surface Area of the Spleen.—At the peak of the effect of an intravenous injection of 0.5 c.c. of 1:1000 adrenalin chloride solution, the average decrease in the lateral surface area of the spleen under nembutal anesthesia was 65.93 per cent, with a range of from 60.81 to 70.49 per cent (Table IV). This demonstrates that, under nembutal anesthesia, adrenalin contracts the spleen in dogs to approximately one-third its preinjection size. Upon exposure of the spleen after laparotomy, we noticed a slight reduction in the size of the spleen, an observation which indicates that the figures given may not be as accurate as they could have been if it were possible to get at the spleen without any manipulation whatsoever.

Comparative Changes in Jugular and Splenic Blood.—In order to compare the effects of adrenalin on the various constituents of blood from the external

TABLE IV. CHANGES IN LATERAL SURFACE AREA OF SPLEEN FOLLOWING INTRAVENOUS INJECTION OF ADRENALIN IN DOGS UNDER NEMBUTAL ANESTHESIA

DOG	LATERAL SURFACE AREA IN SQ. CM.		% DECREASE IN SURFACE AREA
	BEFORE ADRENALIN	AFTER ADRENALIN	
1	162.24	47.88	70.49
2	206.28	80.84	60.81
3	189.00	65.18	65.51
4	213.75	80.66	62.27
5	141.40	43.20	69.45
6	195.70	64.48	67.05

TABLE V. COMPARATIVE CHANGES PRODUCED IN BLOOD WITHDRAWN SIMULTANEOUSLY FROM EXTERNAL JUGULAR AND SPLENIC VEINS DURING SECOND MINUTE AFTER INTRAVENOUS ADMINISTRATION OF ADRENALIN

DOG	HEMOGLOBIN (GM./100 C.C.)				R.B.C. (MILLIONS/C.MM.)			W.B.C. (THOUSANDS/C.MM.)			PLASMA PROTEINS (GM./100 C.C.)		
	JUGU- LAR	SPLEN- IC	DIFFERENCE		JUGU- LAR	SPLEN- IC	DIF- FER- ENCE	JUGU- LAR	SPLEN- IC	DIF- FER- ENCE	JUGU- LAR	SPLEN- IC	DIF- FER- ENCE
			GM.	%									
1	16.2	17.1	0.9	5.55	7.71	8.39	0.68	16.2	20.0	3.8	5.50	5.80	0.30
2	14.4	15.2	0.8	5.55	6.15	7.60	1.45	5.8	8.0	2.2	5.63	5.63	0.0
3	17.1	19.8	2.7	15.84	7.02	7.79	0.77	6.0	10.8	4.8	4.84	4.76	0.08
4	15.2	17.6	2.4	15.78	6.46	7.08	0.62	16.4	17.6	1.2	5.50	5.50	0.0
5	17.3	19.2	1.9	10.98	6.86	7.09	0.23	12.6	12.8	0.2	5.67	5.63	0.04
6	14.8	16.6	1.8	12.16	6.84	7.27	0.43	15.6	16.8	1.2	5.76	5.58	0.18

jugular and from the splenic veins, we took during the operation simultaneous samples from both veins at the time when the spleen was undergoing contraction as a result of an intravenous injection of adrenalin. Detailed data on such blood samples taken from each of the six dogs during the operation is shown in Table V. Blood withdrawn from the splenic vein during contraction of the spleen consequent to adrenalin injection showed an average increase in the red blood cell count of 0.70 million per cubic millimeter, with a range of from 0.23 to 1.45 million per cubic millimeter over the count taken on blood simultaneously withdrawn from the external jugular vein. The concentration of hemoglobin in blood from the splenic vein showed an average increase of 1.7 Gm. per 100 c.c., with a range of from 0.8 to 2.7 Gm. per 100 c.c. over that of blood simultaneously withdrawn from the external jugular vein. Expressed in percentage, the hemoglobin content of blood from the splenic vein averaged 10.98 per cent, with a range of from 5.55 to 15.84 per cent higher than that from the external jugular vein.

The white cell count on blood withdrawn from the splenic vein during contraction of the spleen showed an average increase of 2.2 thousand per cubic millimeter, with a range of from 0.2 to 4.8 thousand per cubic millimeter over that taken from the external jugular. There was no significant difference in plasma protein concentration between blood from the splenic and that from the external jugular vein.

SUMMARY AND CONCLUSIONS

The effects of adrenalin and of nembutal anesthesia on the concentrations of hemoglobin and plasma proteins, and on the red and white blood cell counts,

were studied in intact and splenectomized dogs. The lateral surface area of the spleen was determined under nembutal anesthesia before and after administration of adrenalin. The following conclusions can be made from this study:

1. Intravenous administration of adrenalin increases the concentration of hemoglobin and of the red cell count in circulating blood to a much greater extent in intact than in splenectomized dogs.

2. Nembutal anesthesia causes hemodilution with a reduction in hemoglobin concentration and in the red cell count and a slight reduction in the plasma protein concentration.

3. The size of the spleen in dogs under nembutal anesthesia was decreased about 66 per cent by intravenous administration of adrenalin.

4. The blood squeezed out by the spleen during its contraction under the influence of adrenalin had an average hemoglobin concentration of about 11 per cent higher than that of jugular blood.

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PECTIN EXCRETION STUDIES IN THE HUMAN BEING

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DEMONSTRATION of the efficacy of pectin in increasing plasma volume^{1, 2} and of its therapeutic value in the treatment of shock^{3-5, 6} preceded the development of a method for determining the pectin content of urine and blood. A recent publication gave an analytical method of converting pectin to a pentose and subsequently to furfural which makes it possible to recover 95 to 100 per cent of pectin added in vitro to animal tissues and fluids.⁶

The importance of carrying out pectin excretion studies in the human being is evident from the following considerations: (1) The fate of any fluid injected into the body must be well accounted for before it can be considered innocuous; (2) its rate of appearance and disappearance in blood and urine should be correlated with its physiologic effects; (3) the optimum levels necessary for the production of a desired degree of hemodilution and the relation of the duration of hemodilution to excretion, as well as the relation of the blood to urine levels, should be known; (4) the influence of the shock state offers another problem, and finally; (5) the factor of renal function should also be investigated.

METHODS

In this study, twenty-six patients received between 1,000 and 3,000 c.c. of a 1.5 per cent solution of pectin in saline (buffered before administration)§ intravenously at a rate of approximately one liter in ninety minutes. Ten of these patients were admitted for herniorrhaphies, whereas the remainder had a variety of medical and surgical diseases without involvement of the cardiovascular system, with the exception of three in whom the pectin was administered for the treatment of postoperative shock. Pectin blood levels were determined before the infusion, after administration of each subsequent liter, and at twenty-four-hour intervals thereafter during the period of observation. The

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§The pectin solutions, autoclaved to give an average molecular weight between 40,000 and 60,000 and a viscosity greater than human plasma but less than that of blood, were provided by Frederick Stearns & Co., Detroit, Mich.

samples were drawn in tubes containing ammonium and potassium oxalate. Simultaneously blood samples were drawn in test tubes containing heparin for the determination of hematocrit, hemoglobin, plasma density as index of total protein concentration, and sedimentation rate as previously described.^{1,2} In ten of the twenty-six patients the pectin excretion in the urine was also determined. Patients were asked to empty the bladder before the infusion and this sample was used as the control. Urine samples after administration of each liter of pectin and twenty-four-hour specimens thereafter were collected (with xylene as a preservative). Blood pressure, temperature, pulse and respiration, and any untoward effects were also noted.

The blood sample for pectin analysis was prepared as follows:

Two cubic centimeters of oxalated blood were weighed and then mixed with 13 c.c. distilled water, 2 c.c. of 10 per cent sodium tungstate solution, and 3 c.c. of 0.5 N sulfuric acid. After standing for fifteen minutes and centrifuging for thirty minutes, 2 c.c. of the centrifugate were steam distilled with 5 c.c. of 85 per cent phosphoric acid. Five cubic centimeters of the distillate were mixed with 0.50 c.c. of aniline and 4.5 c.c. of glacial acetic acid, and the intensity of the color developed after standing fifteen minutes in the dark was determined by a photoelectric colorimeter. The urine sample was prepared by mixing 2 c.c. of urine with 10 volumes of 95 per cent alcohol. After an hour of standing the mixture was centrifuged, the supernatant liquid poured off, and the tubes with residue dried to remove alcohol. The dried residue was steam distilled with 85 per cent phosphoric acid and the procedure carried out as for the blood distillate.

From previous work⁶ it was found that the solution of pectin used in this study produced 3.5 mg. furfural per cubic centimeter of pectin sol. Therefore, the control furfural value (per cubic centimeter of urine) was deducted from that found after infusion; the latter multiplied by the volume of urine gave the net or net total furfural value which, when divided by 3.5, gave us the apparent amount of furfural in the urine as pectin sol. The details of the method are described in another publication.⁶

RESULTS

The control value for blood furfural usually ranges from 0.02 to 0.09, with an overall average of 0.05 mg. per gram of blood. Control values in the same patient drawn at one-week intervals were identical. The average blood pectin levels attained immediately after infusion of 1,000 c.c. of a 1.5 per cent pectin solution in twenty-six patients were equivalent to 0.369 mg. of furfural per gram of blood (Fig. 1). The three patients that exceeded these averages most markedly were those treated for postoperative shock.

The difference in the height of the blood pectin level in hospital control subjects and patients in shock is demonstrated by two representative cases in Fig. 4, in which also the higher degree of hemodilution in shock cases is indicated.

The average blood pectin level after a continuous infusion of 2,000 c.c. of the solution was equivalent to 0.59 mg. of furfural per gram of blood, and in

no ease was the rise after the second liter equivalent to the initial rise (Figs. 2 and 3). A single patient given a continuous infusion of 3,000 c.c. attained a blood furfural level of 1.06 mg. per gram of blood, the highest blood level in the series (Fig. 2).

The average of blood pectin levels twenty-four hours after infusion was equivalent to 0.14 mg. of furfural per gram of blood for those given 1,000 c.c. and 0.20 mg. for those given 2,000 c.c. or more. After twenty-four hours the blood levels of those receiving only 1,000 c.c. fell to control values but remained significantly above control levels for seventy-two hours in those receiving 2,000 c.c. or more. (Figs. 2 and 3.)

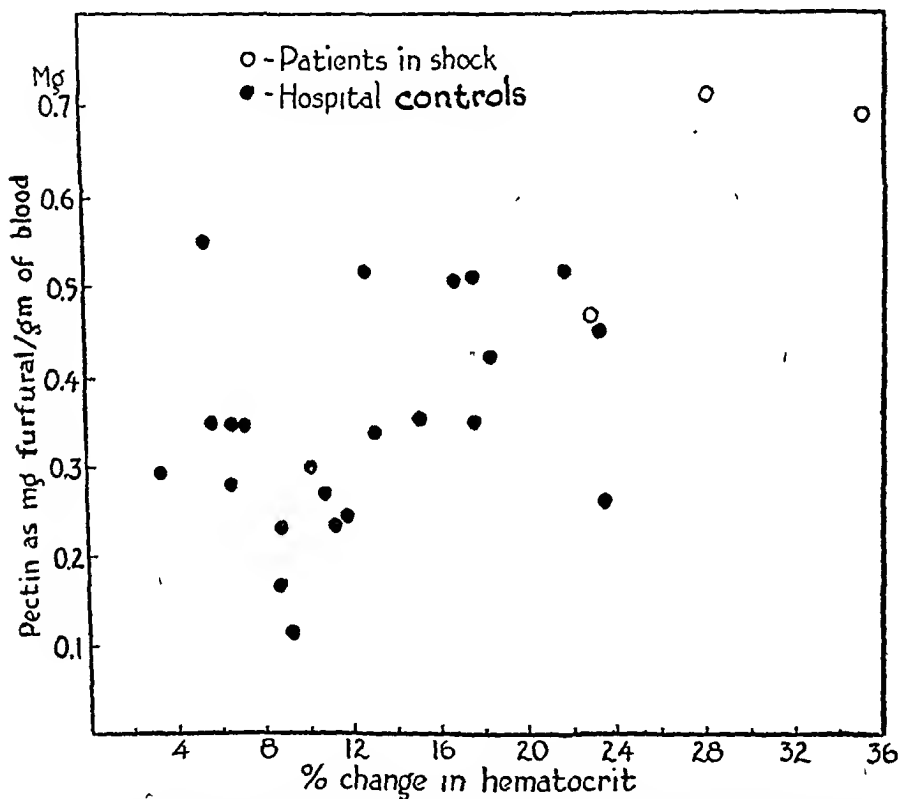


Fig. 1.—Maximal blood levels of pectin obtained by the administration of 1,000 c.c. of pectin to twenty-three patients not in shock (●) and three patients in shock (○) and its correlation with the percentage change in hematocrit thereby produced (as the index of hemodilution). There is some parallelism between hemodilution and the pectin level.

In Fig. 1 is represented a plot of the percentage change in hematocrit, as index of hemodilution, at the end of infusion with the blood pectin level at that time in patients given 1,000 c.c. of pectin solution. There was not a constant relationship between these factors. However, there was a trend for the hemodilution to increase in proportion to the rise in the pectin level; the highest blood levels were seen in cases with maximal hemodilution. This is particu-

larly true in the three patients in shock who showed more hemodilution than did the patients not in shock, as previously observed.³

The control furfural values in the urine of the ten patients examined ranged between .01 and .05 mg. per cubic centimeter of urine with an average of 0.026 mg. per cubic centimeter (Table I). The quantity of pectin appearing in the urine at the conclusion of the intravenous injection of 1,000 c.c. varied considerably in the ten patients, with an average of 151 c.c. (15.1 per cent) of the 1,000 c.c. injected. Those who excreted insignificant amounts during this period usually voided 20 c.c. or less of urine. Those who received a second 1,000 c.c.

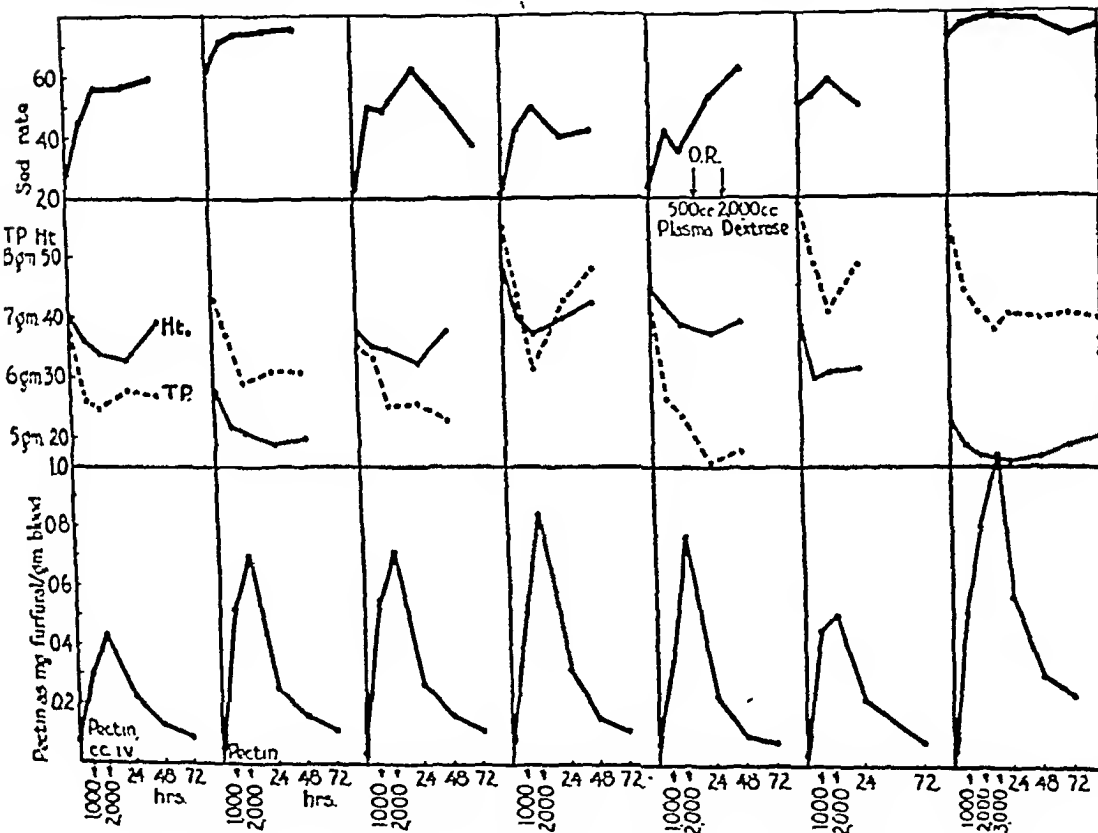


FIG. 2.—Correlation of blood pectin levels (as milligrams of furfural per gram of blood) with hematocrit (Ht.), total protein (Tp.), and sedimentation rates. Changes immediately following the infusion of each liter of pectin and at twenty-four hour intervals thereafter. The pectin level parallels the onset and degree of hemodilution but not with its duration. The sedimentation rate bears a similar relationship.

excreted on an average an additional 138 c.c. during the administration of the second liter (totaling 14.5 per cent of the total pectin injected). At the end of the twenty-four hour period those who had received 1,000 c.c. excreted an average of 256 additional c.c. of pectin (totaling 40.7 per cent excretion of injected pectin). Those receiving 2,000 c.c. excreted an additional 448 c.c., totaling an average of 36.9 per cent excretion of injected pectin. During the forty-eight

TABLE I. VOLUME OF URINE AND PECTIN EXCRETION* IN TEN PATIENTS

PATIENTS RECEIVING 2,000 C.C. PECTIN I.V.												
	1			2			3			4		
	VOLUME URINE (C.C.)	G.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	G.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	G.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	G.C. PECTIN EXCRETED	% PECTIN EXCRETED
Control (mg. furfural)		.02			.01			.02			.01	
After 1,000 c.c.	0	0		230	30	1.5	165	222	11.1	450	317	15.9
After 2,000 c.c.	210	235	11.7	210	28	1.4	120	144	7.2	1,450	137	6.8
After 24 hours	1,470	798	39.9	1,620	463	23.1	990	331	16.5	4,420	202	10.1
After 48 hours	2,820	145	7.2	1,560	107	5.3	1,440	103	5.2	4,000	114	5.7
After 72 hours	2,160	25	1.2	3,120	63	3.1	1,680	33	1.7	2,250	19	1.0
After 96 hours	1,320	8	0.4	2,160	25	1.2	1,170	20	1.0	-	-	-
After 120 hours	1,920	11	0.5	2,220	13	.7	-	-	-	-	-	-
After 144 hours	990	3	0.1	2,880	16	.8	-	-	-	-	-	-
Total		1,225	61.3		745	37.2		853	42.7		789	39.5

*Pectin in urine = $\frac{\text{Gross furfural value}}{3.5}$ (Furfural \times volume of urine)

(Each cubic centimeter of pectin produces 3.5 mg. furfural.)

†During the period of initial infusion of 1,000 c.c. pectin solution, an average 15.1 per cent of the injected pectin already appeared in the urine. At the end of twenty-four hours, an average 38.5 per cent of injected pectin was in the urine, whereas the average total amount of pectin excreted during the entire period of observation was 46.6 per cent.

hour interval the patients who received 1,000 c.c. excreted an average of 51.6 c.c. (an average total excretion now of 45.9 per cent of injected pectin) and those receiving 2,000 c.c., an average of 117 c.c. (an average total excretion of 42.7 per cent at the end of forty-eight hours). Significant excretion during the seventy-two hour interval occurred only in those receiving 2,000 c.c.; thereafter those receiving 1,000 and 2,000 c.c. excretion in both was not considered significant. The total amount of pectin excreted was 47.7 per cent for those receiving 1,000 c.c. and 45.1 per cent for those receiving 2,000 c.c. at the end of the period of observation.

The hemodilution, as judged by hematocrit and total protein, produced in patients given 2,000 c.c. of pectin solution was much more marked and more prolonged than in patients given 1,000 c.c. and therefore results on only the former are presented in graphic form, although the latter showed a similar trend. At the end of twenty-four hours when the pectin (Figs. 2 and 3) excreted in urine of those given 2,000 c.c. was 86 per cent of the total pectin excreted and that of those given 1,000 c.c. was 81 per cent, hematocrit and total plasma protein values were far below control values in the former group and slightly below in the latter. This disparity between the degree and duration of hemodilution and the blood and urine levels of pectin persisted for as long as five days in those given 2,000 c.c., which is the longest period observed. The period of hemodilution persisted beyond the period of significant pectin levels in all cases. This was less striking in the patients given 1,000 c.c. because the hemodilution was less in degree and duration. The increased sedimentation rate (due to pseudo-agglutination) attained its maximal increase at the peak of the blood pectin level, but in those with initial control sedimentation rates close to

FROM 1,000 TO 2,000 C.C. PECTIN SOLUTION INTRAVENOUSLY†

PATIENTS RECEIVING 1,000 C.C. PECTIN I.V.													
1		2		3		4		5		6			
C.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	C.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	C.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	C.C. PECTIN EXCRETED	% PECTIN EXCRETED	VOLUME URINE (C.C.)	C.C. PECTIN EXCRETED	% PECTIN EXCRETED
7	.02	270	.01	300	.04	240	.05	10	.04	210	.04	187	18.7
20	2.0	140	14.0	211	21.1	227	22.7	13	1.3	187	18.7	187	18.7
365	36.5	2,280	37.8	480	12.2	750	18.0	630	24.9	960	24.1	241	24.1
54	5.4	2,100	6.6	1,380	35	1,080	43	1,320	61	1,260	51	51	5.1
22	2.2	1,320	3.0	960	6	1,440	21	780	9	1,170	20	2.0	2.0
15	1.5	2,940	3.4	480	7	2,400	3.4	900	5	3,360	0	0	0
14	1.4	-	-	-	-	-	-	960	6	-	-	-	-
490	49.0	648	64.8	381	38.1	505	50.5	343	34.3	499	49.9	49.9	49.9

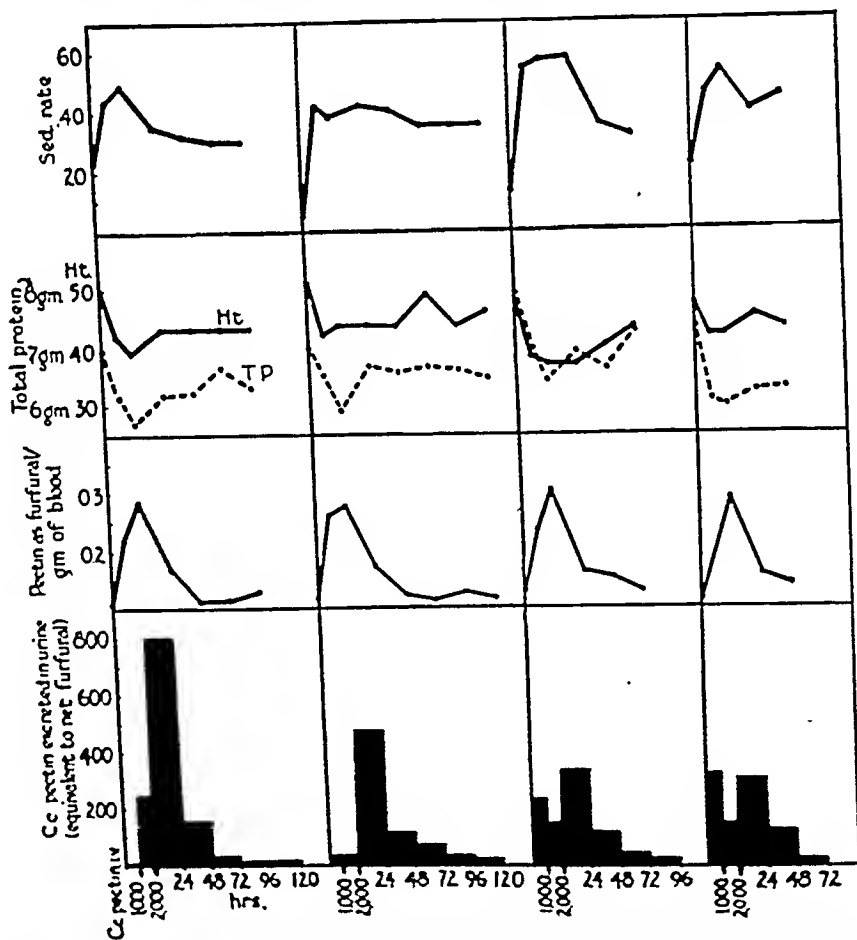


Fig. 3.—Correlation of blood levels and urinary excretion of pectin with changes in hematocrit (Ht), total protein (Tp), and sedimentation rate in four patients given 2,000 c.c. of pectin in saline. The rate and quantity of pectin excreted varies considerably, although blood levels are rather constant. At the end of twenty-four hours, when an average of 45 per cent of excretable pectin has been eliminated, hem dilution is still marked.

normal, the elevated rate persisted long after the pectin blood level has returned to control levels. The relationship of the sedimentation rate to pectin excreted in urine was about the same.

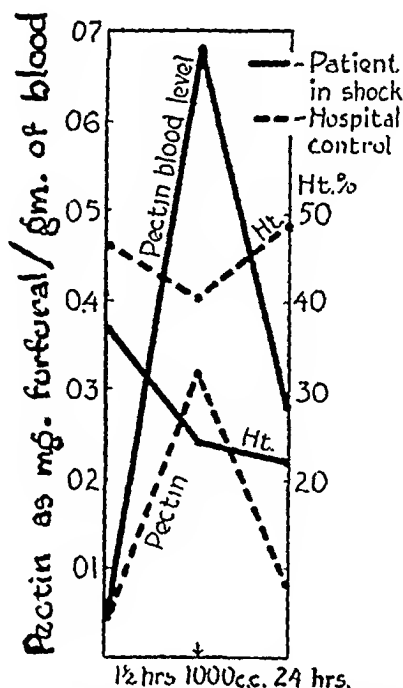


Fig. 4.—Comparison of hemodilution and pectin blood levels in patient in shock (—) and a patient not in shock (---). Both were given 1,000 c.c. pectin solution. The blood pectin level of the patient in shock, as well as the degree and duration of hemodilution, far exceeds that in the patient not in shock; the blood level is also significantly higher twenty-four hours later. The patient in shock presented an initial hematocrit (Ht.) of 37 per cent and total protein of 6.05 Gm. per cent which eliminates the possibility of hemoconcentration. A more likely explanation of the difference is impaired renal function.

DISCUSSION

The recent development of a chemical assay method for pectin in blood and urine provides the basis for studies which should have preceded the clinical administration of pectin. Such studies will shed new light on the clinical indications for the intravenous administration of pectin.

The basic phenomenon in shock is the reduction of blood volume. Pectin solution is a macromolecular plasma substitute used to increase plasma volume because the large pectin molecule keeps the injected fluid for a considerable time in the circulatory bed. The measurement of the plasma volume, which offers some technical difficulty, is substituted in clinical studies by the determination of the hemodilution measured by the reduction of the hematocrit, the hemoglobin concentration, and the total plasma protein concentration. In previous studies it was demonstrated that pectin produces considerable hemodilution in patients not in shock^{1, 2} but a still more marked hemodilution in patients in shock.³ The degree of hemodilution initially produced by the administration of the pectin solution is dependent upon the amount of pectin administered, as shown by the

rough parallelism existing between the degree of hemodilution and the pectin level obtained. In this sense the pectin level is an index of the therapeutic effect obtained by pectin.

The blood pectin levels, following the infusion of a given amount of pectin solution, are rather consistent in hospital controls. They increase if more pectin is given. However, in patients in shock, the pectin level, after administration of 1,000 c.c., is on an average higher than in the controls after administration of 2,000 c.c. This agrees well with the higher degree of hemodilution obtained in patients in shock. The question "Which is the optimal pectin level?" is as yet not settled. Not only the concentration, but also the duration of the pectin concentration in the blood, is directly related to the amount of pectin introduced. The difference in shock is probably caused by the difference in urinary excretion of pectin. Renal excretion is known to be decreased in the shock state.

Whereas a rough parallelism exists immediately after administration of pectin between the pectin level and the hemodilution produced, this relationship deviates later on. The plasma pectin level decreases much faster than the hemodilution disappears, a fact which suggests that as pectin disappears, other substances maintain the hemodilution. Similarly, the effect pectin has upon the red cell, namely, the pseudo-agglutination as indicated by the rise of the sedimentation rate, is maintained longer than the pectin level.

The urinary excretion of pectin deserves interest from two viewpoints: First, if an unphysiologic substance is introduced into the body, one prefers that it be disposed of quickly; second, the relation between blood level, urinary excretion and the therapeutic effect as evidenced by the hemodilution, gives information to the mechanism of this effect. The excretion of pectin starts with the administration of pectin and a considerable amount is excreted by the end of the infusion. However, this fast excretion comes to a standstill within three days, by which time approximately 45 per cent of the originally introduced pectin has left the body. Since a third of the total amount of pectin excreted has left the body during the infusion, one can assume that a great part of pectin is apparently removed from the reach of the kidney within a short time. There is a remote possibility (unproved thus far) that some of the pectin may be broken down to simple sugar molecules and may thus escape the alcohol precipitation involved in the preparation of urine samples in the present method for pectin analysis.

Recently we have demonstrated in human subjects and in rabbits the deposition of a peculiar material in the pulp of the spleen and around the glomerular loops of the kidney associated with a proliferation of the reticulo-endothelial cells of the spleen and a proliferation and vacuolization of the Kupffer cells of the liver. Since the spleen was markedly enlarged in rabbits and especially in patients with nephrosis, most of the unexcreted (and/or metabolized) material can be assumed to have been deposited in the spleen. However, its nature is not clear in view of the fact that chemical analyses by Joseph, Bryant, and Palmer⁷ failed to detect an increased amount of substances giving a furfural reaction in the organs. However, the presence of this

material may be an indication that retained pectin or a derivative of it is responsible for changes in the organs.

It should be stressed, however, that those findings were encountered only after administration of amounts of pectin far in excess of those used in shock. It should further be emphasized that the possibility exists that other pectin solutions act differently. Hartman⁸ did not observe such anatomic changes after administration of equivalent amounts of pectin, and we have to reserve judgment as to whether an otherwise prepared pectin solution would be retained in tissues to a lesser degree and lead to similar anatomic changes.

At a time when the hemodilution is still of considerable degree, the blood level has already markedly decreased and the greater part of the discardable part has been excreted with the urine; the greater part of the nondiscardable portion is at this time obviously out of the circulation. Heretofore one of the prime essentials of an effective plasma substitute has been considered the property of being retained within the circulation for a long period of time. The present findings show that a substance like pectin may produce hemodilution even longer than it is present in the blood stream. Any substance which could induce this phenomena of prolonged hemodilution after its disappearance from the circulation has considerable promise. We ascribe it to the ability of a macromolecular substance to draw tissue fluid into the circulation. This tissue fluid, which replaces the fluid leaving the circulation with pectin, presumably contains proteins which are available in large quantities in the labile protein stores of the body. The term "autotransfusion" can be used to describe this phenomena.

SUMMARY

1. Using a chemical assay for pectin recently described, blood pectin levels and urinary pectin excretion following the administration of pectin solution have been described.

2. The blood pectin levels are proportionate to the quantity of pectin administered. They are higher in patients in shock, probably due to depressed renal function.

3. The degree of initial hemodilution is somewhat parallel to the level of pectin in the blood; however, hemodilution and the increased sedimentation rate (pseudo-agglutination) produced by pectin are maintained much longer than blood pectin level.

4. The pectin excretion starts rapidly and decreases after the first day. However, by the analytical methods available, less than one-half of the injected pectin is accounted for in the urine, indicating that more than one-half of the pectin injected is quickly removed from the reach of the kidney, some of which is probably deposited in certain organs.

5. The maintenance of the hemodilution beyond the period of significant blood concentration of pectin and of urinary elimination of the great amount of excreted pectin indicates that the prolonged hemodilution is not due to pectin but possibly to labile tissue proteins.

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THE USE OF THE COPPER SULFATE METHOD OF HEMOGLOBIN ESTIMATION FOR SCREENING BLOOD DONORS

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THE number of methods which are available for the estimation of hemoglobin values, and which are at the same time applicable to mass production techniques, is limited. Individual centers of the American Red Cross Blood Donor Service have been called upon at times to process as many as 1,100 donors daily, and a hemoglobin estimation must be made on each donor. Such volume necessarily requires a method which is both fast and accurate and which can be carried out by relatively untrained personnel.

The methods which are the most accurate and which are in common use in most laboratories and offices, such as the Dare, the Sahli, the Newcomer, and certain other colorimetric techniques, invariably require a measured amount of blood which, in turn, requires relatively complicated equipment and trained personnel. The Tallqvist method, which is admittedly the simplest and most rapid method, can be used only with difficulty when one individual is required to make from 100 to 200 such comparisons within a period of a few hours. There is not only considerable error in the method per se, but there is also the additional factor of ocular fatigue with such a large number of tests. It was necessary, however, to use this method during the first two years of operation of the Blood Donor Service since no other method which met the requirements was available.

In December, 1943, a simple method for determining the specific gravities of whole blood and plasma using a copper sulfate solution was devised at the Rockefeller Institute.† This method is based on the fact that a drop of blood or plasma dropped into a precision-made solution of Cu SO_4 of known specific gravity will become encased in a sac of Cu proteinate and will behave in a certain way relative to that solution, dependent upon the density of that drop. If a graded series of Cu SO_4 solutions is used, the actual specific gravity of the blood and plasma may be determined and from these, by calculation from line charts, the hemoglobin, plasma protein, and hematocrit values can be obtained. For the purposes of the Red Cross Blood Donor Service, however, a single solution of specific gravity 1.052, corresponding to 12.3 Gm. of hemoglobin, permits the acceptance or rejection of a donor without obtaining the exact amount of hemoglobin in that donor's blood. In other words, if a drop of the donor's blood sinks in the solution, the donor is acceptable; if the drop rises or remains suspended, the individual's hemoglobin is less than the acceptable standard; that is, less than 12.3 Gm. This paper describes the technique and analyzes the results as obtained at the Blood Donor Center of the New York Chapter of the American Red Cross with the use of this method.

From the New York Blood Donor Center of the American Red Cross Blood Donor Service. Received for publication, Sept. 20, 1945.

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†Phillips, R. A., and others: Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, Bull. U. S. Army M. Dept. 7: 66, 1943.

TECHNIQUE

The equipment necessary to make the test is illustrated in Fig. 1. It consists of a lancet for making the finger puncture, capillary tubes with rubber bulbs attached, and Cu SO_4 solution in large stock bottles and in a smaller vial for actual use. The correct position for expelling the drop is also shown.

1. The tip of the fourth or ring finger is thoroughly cleansed with alcohol and then wiped *dry* with sterile gauze. The finger is punctured, somewhat forcibly, with a lancet or special needle and the first drop of blood obtained is wiped off.

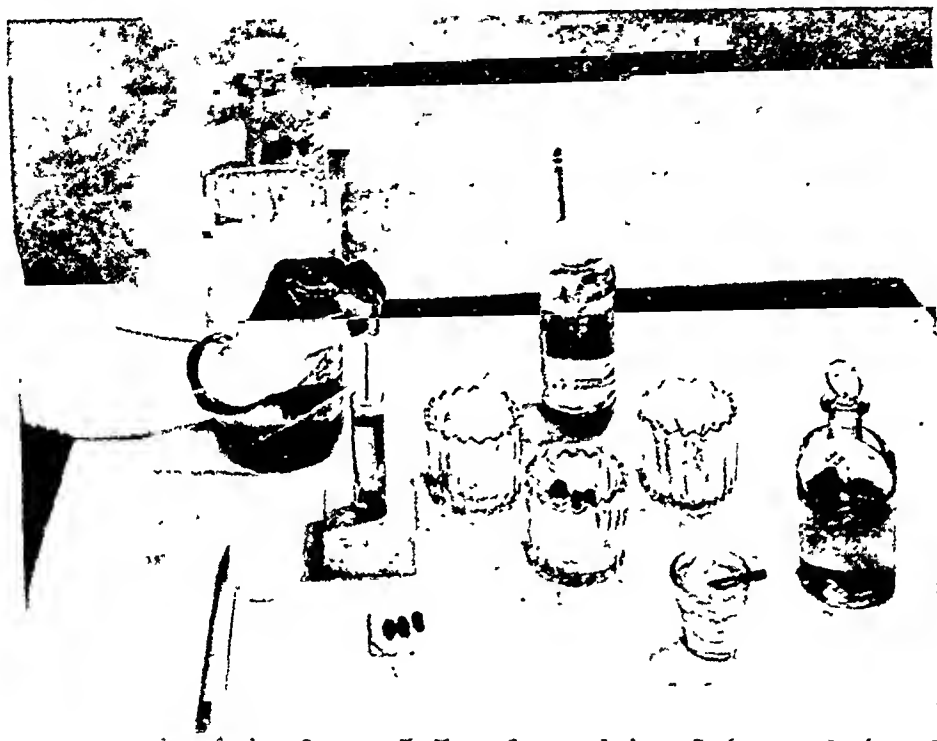


FIG. 1.

2. A second drop of blood is squeezed and allowed to run up into a capillary tube of about 1 mm. bore with a small rubber bulb at one end.*

3. The blood is ejected into the solution of Cu SO_4 by gentle pressure on the bulb, and the behavior of the drop is observed. The initial momentum of the drop is lost within a few seconds and the drop then either (1) begins to rise, (2) remains stationary for another ten to fifteen seconds, or (3) continues to fall in the solution. If either the (1) or (2) condition obtains, the donor is rejected; if (3) obtains, the donor is accepted.

While the technique itself is simple and rapid and can be easily carried out by relatively unskilled personnel, it has been apparent since the intro-

*The use of a capillary tube and rubber bulb instead of a syringe or pipette was suggested by Dr. William Thalhimer, associated with the American Red Cross Blood Donor Service in 1944 as Associate Technical Director.

duction of the method in Red Cross centers in April, 1943, that the following precautions should be taken to avoid inaccuracies and errors of interpretation.

1. Theoretically, one test may be done per cubic centimeter of Cu SO_4 solution. If a small vial of from 25 to 30 c.c. of solution is used, as in Fig. 1, careful count must be kept of the tests made and fresh solution must be used after about twenty tests. The color of the solution may shift from blue to green during the course of the tests and may develop a certain degree of turbidity because of the presence of suspended unlaked cells, but the gravity of the solution is not perceptibly affected by these changes.

2. The finger used must be *thoroughly* cleansed with alcohol and then dried, and the first drop of blood obtained after the puncture should be wiped off. Even a minute amount of dirt, grease, or alcohol remaining on the finger alters the behavior of the drop.

3. The blood drawn into the capillary tube should be free of air bubbles and should form an intact drop in the solution.

4. The surface of the Cu SO_4 solution should be kept free of fragments of blood. These are removed by shaking the vial or by using a wooden applicator stick.

5. In interpreting the results, it should be remembered that the drop will have a certain initial momentum downward in the solution. When this initial force has subsided, the drop may continue downward, remain suspended, or rise. It is, therefore, the behavior of the drop within the ten seconds *after* the initial momentum has subsided that is of significance.

6. Care should be taken to keep the Cu SO_4 solution well covered, preferably with an airtight stopper, when not in use. If crystals of Cu SO_4 form around the top of the bottle, or on any device used for pouring, a fresh bottle should be taken and the equipment cleaned.

7. According to Phillips and his associates, there is an inherent error of ± 2 per cent in the method when blood with cells of normal hemoglobin content is being tested. In abnormal cases, the error may be ± 4 per cent. The estimation of hemoglobin *from whole blood* by this method is based on the assumption that the plasma protein concentration is within normal limits. However, high plasma protein values may cause a plus error in the hemoglobin estimation, and low plasma protein values may give a low estimate of the hemoglobin. Since it would be inadvisable to accept a donor with a low plasma protein value, such an "error" in the hemoglobin estimation is an advantage rather than a fault.

RESULTS

Before the method was put into routine use in February, 1944, and during the period of instruction for the personnel, a comparison was made on 632 donors using the Cu SO_4 and Evelyn photo-electric cell method. Ideally, comparisons of results with the Cu SO_4 technique should be made against the oxygen capacity method; however, since this was not possible, the comparisons were made with an Evelyn colorimeter which had in turn been checked with samples tested by the oxygen capacity method for hemoglobin estimation. In

this series of 632 donors, 108 (or 17.0 per cent) were unnecessarily rejected; that is, the Cu SO_4 reading was below the requisite 12.3 Gm., whereas the Evelyn reading was 12.3 Gm. or above. In this same group, there were five donors (or 0.9 per cent) who were accepted in error; that is, the Cu SO_4 reading indicated over 12.3 Gm., but the actual hemoglobin values were below 12.3 Gm. by the Evelyn colorimeter. Although the errors in this series might have been said to be "on the safe side," the results of the comparison indicated that the staff was apparently not carrying out the technique correctly and steps were taken to remedy this. It was found that the errors made were chiefly those noted previously; for instance, alcohol remaining on the finger, air bubbles in the tube, carelessness in handling the solutions. A second comparison series on 1,012 donors was carried out in April, 1945. In this group, twenty-two (or 2.17 per cent) were rejected in error because they had an Evelyn value over 12.5 Gm., and twenty (or 1.98 per cent) were accepted in error because they had an Evelyn reading below 12.3 Gm. (Using a solution of specific gravity 1.052, hemoglobin values between 12.3 and 12.5 Gm. were considered to be within the "critical range"; for instance, for Evelyn values within this range the behavior of the drop may, within the limits of error, follow any one of the three courses.)

TABLE I

	FEBRUARY, 1944	APRIL, 1945
Number tested	632	1,012
Number rejected in error (Cu SO_4 less than 12.3 Gm.)	108	22
Per cent	17.0	2.17
Range of error by Evelyn method (Gm.)	12.5 to 14.2	12.5 to 13.8
Number accepted in error (Cu SO_4 more than 12.5 Gm.)	5	20
Per cent	0.9	1.98
Range of error by Evelyn method (Gm.)	11.0 to 12.2	11.0 to 12.2

In Table I the complete results of the two series of comparisons are given.

Over a period of a year at the New York Center, the percentage rejection rate for prospective initial donors on the basis of hemoglobin, using the Cu SO_4 method, is given in Table II.

TABLE II

	NUMBER TESTED	PER CENT REJECTED
Male	83,460	0.25
Female	101,004	7.9

TABLE III

	NUMBER TESTED	MEAN HEMOGLOBIN VALUE (Gm.)	NUMBRL. BELOW 12.3 Gm.	PER CENT BELOW 12.3 Gm.
Male	2,019	15.8	5	0.24
Female	3,527	13.76	271	6.95

On the basis of almost 6,000 determinations made by the Evelyn photo-electric colorimeter on male and female initial donors over a period of two years at the New York Center, the values given in Table III were obtained.

It will be seen that in this comparison again the Cu SO_4 method compares very favorably with the Evelyn determination. The rejection rate by either method is essentially the same.

SUMMARY

The Cu SO_4 method of hemoglobin estimation has been in use in the New York Center of the American Red Cross Blood Donor Service for over a year and one-half. Under the conditions obtained in such a mass blood collection program, it has provided a simple and rapid method of determining whether or not a donor's hemoglobin is above or below the acceptable minimum of 12.3 Gm. The test may be done by unskilled volunteer personnel after a brief course of instruction. Testing individuals with hemoglobin, hematocrit, and plasma concentration values presumably within normal limits, the error in the method should not be more than ± 2 per cent. The experience in the New York Blood Donor Center, where it may be assumed that individuals do not present themselves for a donation unless they believe themselves to be in reasonably good health, has indicated that such is the case. The error is kept at the irreducible minimum, however, only when rigid attention is paid to the details of the technique and to the proper interpretation of the behavior of the drop. The test as performed in Red Cross Blood Donor centers would be easily applicable to any blood donor program or for "screening" purposes in connection with physical examinations where it would be of value to know whether or not an individual hemoglobin was above or below a certain standard. With the use of graded solutions of Cu SO_4 , spaced at various gravity levels, a more accurate estimation could be made if desired.

CONCLUSIONS

1. The use of the Cu SO_4 gravity method of hemoglobin estimation of Phillips and his associates, as used at the New York Center of the American Red Cross Blood Donor Service, is described.

2. Certain precautions are noted and certain details of the technique are emphasized in order to obtain maximum accuracy and correct interpretation of the results.

3. A comparison is made of the results obtained by this method and by an Evelyn photo-electric cell colorimeter on a large series of blood donors.

STUDIES ON CULTIVATION OF RICKETTSIAE IN EGGS

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THE work of Goodpasture¹ with the cultivation of viruses on the chorio-allantois of the developing chick embryo has been of inestimable value to bacteriologists whose interest lies in the field of viruses and rickettsiae. It marked the beginning of large scale work with these agents and has made possible the development of vaccines against some of the world's most devastating diseases. Zia² and Bengtson and Dyer³ succeeded in obtaining multiplication of typhus and spotted fever rickettsiae, respectively, using Goodpasture's technique. Barykine and co-workers⁴ and Cox⁵ showed that better results could be obtained if inoculation were made directly into the yolk.

Although many investigators have been using the yolk sac method of rickettsial cultivation, detailed results, except those from China, have not been published.^{6,7} In most new fields, much of one's knowledge is acquired by trial and error methods. The cultivation of rickettsiae in eggs has been no exception. The work detailed in this article on methods of inoculation, susceptibility of chick embryos, neutralization tests in eggs, and soluble antigen experiments is reported in the hope that it may be of use to workers in this field.

Methods and Materials.—The rickettsial strains employed consisted of the Breinl epidemic and the Wilmington murine strains of typhus* and the Bitter Root* and the Cape Cod† strains of spotted fever. The Cox technique of inoculation was used throughout.⁵ Eggs from white leghorn hens were employed to facilitate candling. These eggs were from pullorum-tested flocks and were purchased at six or seven days' incubation. Seven-day-old embryos were used, except where noted. On arrival, the eggs were unwrapped and placed on wooden boards made to accommodate forty or eighty eggs, air sac end uppermost. Prior to inoculation, dead or nonfertile eggs were candled out. Iodine was applied over an area of about 1 cm. in diameter and allowed to dry. A hole was then made with a moto-tool‡ or regular dental drill, the burr being adjusted so as to puncture the shell but not the shell membrane. The shell dust was removed with 70 per cent alcohol. The egg inoculations were performed with regular 10 c.c. or automatic 2 c.c. syringes and 20 gauge needles, 1½ inches long. The eggs were sealed with paraffin and incubated at from 34 to 35° C. The inoculum was prepared by shaking yolk sacs in saline in the presence of glass beads. Cultures were taken before, during, and after inoculation. All yolk sac and vaccine smears were stained by the Macchiavello technique,⁹ with the simple modification of using sterile distilled water without neutralization as diluent for the basic fuchsin. Methylene blue was used for smears of sterility tests. In

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*Obtained through the courtesy of the National Institute of Health, Bethesda, Md.

†Strain isolated from the first case in Massachusetts.

‡Dremel Manufacturing Co., Racine, Wis.

all experimental work and in selecting yolk sacs to be used as inoculum in vaccine preparation, the piece of yolk sac to be examined was removed at its point of attachment to the embryo. This technique was adopted in an effort to approach some sort of uniformity in the examination of smears. As a basis for comparing eggs, it is more exact than when pieces are removed at random. Yolk sacs to be used in vaccine preparation were always selected in advance. From two to six yolk sacs, depending on the richness of the individual smears, were placed whole in bottles containing glass beads. They were shaken and about 0.5 c.c. of the concentrated yolk removed for culture. The bottles were then placed in a chest maintained at from -40 to -60° C. with solid carbon dioxide. Yolk sacs kept under these conditions were found to remain potent for seventeen months, the longest interval tested to date.

We have not found the problem of contamination to be a serious one. Cultures of 1,123 pools, representing, 3,963 yolk sacs grouped in lots of from two to six, revealed that forty-four or 3.9 per cent of the lots were contaminated. If, as was likely, only one yolk sac in each lot had been at fault, the figure for contamination would have been about 1 per cent. We are inclined to attribute most contamination to faulty technique, since as many as fifty-seven yolk sacs cultured individually the same day from the same lot of typhus-infected eggs showed no growth.

Prior to use, the yolk sacs were thawed quickly in water at a temperature of from 40 to 45° C., thoroughly shaken, and diluted with the required amount of salt solution. Pieces of membrane were removed with a bent capillary pipette and the eggs injected with 0.5 or 1 c.c. of inoculum. For routine purposes, one candling of inoculated eggs at the end of forty-eight hours (for spotted fever) or seventy-two hours (for typhus) was considered sufficient to remove those dead of trauma. Subsequently, eggs were candled daily until large numbers of deaths occurred, at which time they were candled several times a day if possible. With the typhus strains, some deaths occurring on the third day, even with high dilutions of inoculum, are due to typhus. However, we have found that the elimination of these eggs is no loss, since the yolk sacs are still small and hard to harvest and do not materially add to the quality of the vaccine. (It is obvious that deaths on the third day in eggs that have received a very heavy typhus inoculum are significant, but for practical reasons these deaths should be avoided in large scale vaccine preparation.)

Yolk sacs to be tested in mice for toxin were either frozen whole or diluted with milk, as suggested by Topping,¹⁰ for the preservation of infected tissues. In the latter case, a 10 per cent suspension was made. The material was centrifuged, decanted, and distributed in small samples in vaccine vials as recommended by Bengtson.¹¹ It was then frozen until needed. We have found canned evaporated milk very satisfactory and more convenient to prepare than the skimmed milk recommended by Topping. The contents of a can of milk were diluted with an equal amount of sterile water. Sufficient N/1 NaOH solution was added to bring the pH up to about 7.5. Since canned milk is sterile, no autoclaving was required.

For dilution experiments in eggs and guinea pigs, 10 per cent infected yolk sac suspensions in saline were prepared, and further dilutions were made from these. Inoculation was then carried out as fast as possible by two operators to prevent deterioration of rickettsiae.

Guinea pig inoculations were performed by administering 1 c.c. of inoculum by the intraperitoneal route to male animals. Mice were vaccinated by the intraperitoneal route¹² and received live yolk sac material by the same route or intravenously.

Age of Embryos and Methods of Inoculation.—In published work on the use of fertile eggs for the cultivation of rickettsiae, embryos of ages varying from 2 to 11 days have been recommended.^{4, 5, 7, 13} It was felt that the older the embryos were at the time of inoculation, consistent with good results, the greater would be the weight-yield of yolk sac and the fewer the early deaths from trauma or overwhelming infection. However, with increasing age, the embryos became more resistant to infection. Experiments undertaken to find the oldest embryo that would give uniformly good results showed that it was possible to infect eggs that had been incubated eight days quite successfully with the two typhus strains. When 9- or 10-day-old embryos were used, good results could be obtained but a more concentrated inoculum was required. When large numbers of eggs were inoculated, it was found convenient to use embryos of an age that could be killed with high dilutions, since fewer yolk sacs were required as inoculum.

Older embryos (from 8 to 9 days) were found to be most useful in spotted fever experiments. In egg-adapted strains of both mild and virulent spotted fever, death occurred in about three days when 6- or 7-day-old embryos were used. Yolk sacs were small, friable, and difficult to harvest. The use of 8- or 9-day-old embryos helped to remedy these conditions. At 10 days, embryos were found to be too insusceptible for routine use.

In an effort to reduce the percentage of traumatic deaths, the embryo's position in the egg was marked during the candling prior to inoculation in several lots of eggs. Duplicate sets receiving the same inoculum were not so marked. It was found that marking the embryos resulted in a somewhat lower mortality. Comparisons were also made between lots of eggs that had been inoculated by the same operator with standard 10 c.c. syringes and with automatic 2 c.c. syringes. With the former, traumatic deaths were 3 or 4 per cent, whereas with the latter type of syringe they were from 18 to 24 per cent. The use of the automatic syringe eliminates the need of filling and cleaning syringes so that if time saved is an important factor, the automatic syringe is the one of choice.

Temperature of Incubation.—Eggs infected with all four rickettsial strains were incubated at from 34 to 35° C. routinely. With experimental lots, it was observed that in typhus-infected eggs, death of embryos occurred at least twenty-four hours earlier than when a temperature of 37° C. was used (Table I). Eggs incubated at 37° C. required a heavier inoculum to kill the embryos than those incubated at 34° C. It would appear that at 34° C., a temperature far below the optimum for healthy development of normal chick embryos, several factors may be involved which contribute to better multiplication of typhus rickettsiae. The embryo's resistance is lowered and rickettsial multiplication can proceed

TABLE I. EFFECT OF TEMPERATURE OF INCUBATION ON TIME OF DEATH OF INFECTED CHICK EMBRYOS*

DAY AFTER INOCULATION	NUMBER OF EGGS DEAD†	
	TEMPERATURE OF INCUBATION	
	34° C.	37° C.
1 to 3	0	0
4	1	0
5	2	0
6	3	0
7	4	5
8	-	4
9	-	1

*Inoculated with 1:400 dilution of murine typhus yolk sac.

†Ten eggs in each group.

more readily than at 37° C., or as has been demonstrated in guinea pigs, rabbits,¹⁴ and mice,^{15, 16} low temperatures favor rickettsial infection of the host. The incubation of infected typhus eggs at 34° C. is advisable in large scale vaccine preparation. It can be readily seen that if a more dilute inoculum is required when eggs are incubated at this temperature, fewer yolk sacs need be prepared as inoculum, thereby reducing labor and the risk of contamination. In the case of spotted fever, the infection is apparently so overwhelming that death of the embryo occurs at the same time whether incubation at 34° C. or 37° C. is used, but smears richer in rickettsiae are obtained from eggs incubated at the former temperature.

Dilution and Diluents.—Experience showed that it was possible, within limits, to bring about death of infected eggs at the time desired. Even under optimal conditions, however, there was a variation in the susceptibility of chick embryos which could not be overcome. When large numbers of eggs were inoculated, aside from those dying of trauma, there were nearly always a few that died early, often showing very few rickettsiae (perhaps overwhelmed by toxin) and a few resistant ones that died late. The great majority died within a twenty-four-hour period (that is, on the fifth and sixth days, or on the sixth and seventh days after inoculation). This point is illustrated in Table II.

The numbers of rickettsiae seen in the smears of the yolk sacs to be used for inoculation determined the amount of diluent to be added. For routine purposes, we found it practical to use for typhus passage only those yolk sacs showing numerous rickettsiae and designated 4-plus or better and to dilute them

TABLE II. NORMAL VARIATION ENCOUNTERED IN TIME OF DEATH OF TYPHUS-INFECTED CHICK EMBRYOS

DAY AFTER INOCULATION	NUMBER OF EGGS DEAD	
	EPIDEMIC TYPHUS*	MURINE TYPHUS†
	60 EGGS	100 EGGS
1 to 3	3	4
4	0	4
5	3	18
6	21	72
7	28	2
8	4	-
9	1	-

*Inoculated with 1:300 dilution of infected yolk sac.

†Inoculated with 1:200 dilution of infected yolk sac.

TABLE III. EFFECT OF pH OF SUSPENDING MEDIUM ON TIME OF DEATH OF INFECTED EGGS*

DAY AFTER INOCULATION	NUMBER OF EGGS DEAD	
	DILUENT	
	NaCl pH 6.0-6.2	PHOSPHATE BUFFER pH 7.4
1 to 3	0	1
5	13	12
6	6	6
7	1	1

*Inoculated with 1:200 dilution of epidemic typhus yolk sac.

with from 200 to 500 c.c. of physiologic salt solution. In the case of spotted fever where smears never approached the richness of typhus yolk sacs, from 50 to 200 c.c. were used. No difference in result was seen when a 1:100 or a 1:200 dilution, for example, was employed, or similarly, when 0.5 or 1 c.c. was used as inoculum. Fivefold dilutions of a given inoculum were required to bring out a noticeable effect on the dying time of the embryo.

Because of the fragility of rickettsiae suspended in physiologic saline, it was thought that dilution of yolk sacs in a buffer of a higher pH than the saline usually employed might keep the rickettsiae in better condition during the time of inoculation. Accordingly, yolk sacs were shaken in 5 c.c. of saline and portions of this suspension further diluted with saline and Sørensen's phosphate buffer, respectively. As will be seen from the data in Table III, the time of death in two groups of twenty eggs was not influenced by the different pH values of the suspending solutions. Smears of the yolk sacs were of similar richness in both groups. The same results were obtained when buffered saline and physiologic saline were compared.

Comparison of Guinea Pig and Chick Embryo Susceptibility.—The guinea pig is not a highly susceptible animal as far as the two types of typhus are concerned. Virulent spotted fever strains, on the other hand, kill a large proportion of inoculated animals. We have attempted to correlate the susceptibility of the chick embryo and the guinea pig by inoculating them with the same dilutions of yolk sac material from eggs infected with murine and epidemic typhus and Bitter Root spotted fever. With murine typhus, the limit of infectivity appeared to be the same for eggs and guinea pigs, but elevation of temperature was noted in the guinea pigs from twenty-four to forty-eight hours before eggs of the same dilution died. In one experiment, the highest dilution that caused death of only a portion of the eggs (1:100,000) also produced fever in only some of the inoculated pigs. However, those having no temperature were found to have been immunized or to have had an inapparent infection, since serum obtained three weeks after inoculation agglutinated murine rickettsial antigen in high titer.¹⁷ When epidemic typhus inoculum was used, chick embryos were more susceptible than guinea pigs in that the highest dilution employed that resulted in death of embryos produced no temperature whatever in any of the guinea pigs inoculated. In a typical experiment, seven of eight eggs inoculated with 1:100,000 dilution of yolk sac died, whereas the guinea pigs receiving the same dilution showed no fever nor did they develop agglutinins for epidemic antigen, showing that an insufficient number of rickettsiae was present even to produce agglutinins. Simultaneous inoculation into

eggs and guinea pigs of Bitter Root strain spotted fever yolk sac dilutions produced results similar to those obtained when epidemic typhus inoculum was used. For example, the highest dilution producing death of chick embryos in one experiment was 1:10,000. At this end point, guinea pigs showed no elevation of temperature or other signs of spotted fever. From the foregoing experiments, it would appear that the chick embryo is more susceptible to infection with epidemic typhus and virulent spotted fever than is the guinea pig. This fact might be made use of in the isolation of strains from the blood of human cases of these diseases where, particularly in mild cases, efforts to isolate strains by guinea pig inoculation are often fruitless.

Neutralization Tests in Eggs.—Evidence of neutralization using mixtures of immune serum and infectious agent introduced into eggs has been obtained for certain viruses and bacteria: vesicular stomatitis,¹⁸ equine¹⁹ and St. Louis encephalitis,²⁰ keratoconjunctivitis,²¹ and mumps²² viruses; typhoid,²³ influenza²⁴ and the tubercle bacillus.²⁵ It was felt that if neutralization of typhus and spotted fever rickettsiae by immune serum could be demonstrated in the chick embryo, a simple test would be provided for the study of neutralizing antibodies in the serum of convalescent men and animals. To this end, potent immune globulins prepared from the serum of rabbits infected with typhus or spotted fever were mixed in vitro with dilute infectious yolk sac material for from 20 to 30 minutes prior to the inoculation of eggs. Controls with an equivalent amount of normal serum were set up at the same time. As will be seen from the results of two typical experiments summarized in Table IV, no evidence of neutralization was obtained with either rickettsial antiserum. In this and other

TABLE IV. NEUTRALIZATION TESTS IN EGGS* WITH EPIDEMIC TYPHUS AND SPOTTED FEVER GLOBULINS

DAY OF DEATH OF EGGS†	EPIDEMIC TYPHUS		SPOTTED FEVER	
	0.1 C.C. GLOBULIN	0.5 C.C. NORMAL SERUM	0.1 C.C. GLOBULIN	0.5 C.C. NORMAL SERUM
1 to 2	1	0	1	0
3	0	0	0	0
4	0	0	2	4
5	0	0	5	4
6	3	4	-	-
7	4	4	-	-

*Inoculated with 1:300 dilution of epidemic typhus or 1:300 dilution of spotted fever yolk sac.

†Eight eggs in each group.

experiments, there was no delay in the death of the embryos, and smears were as rich in rickettsiae as were those from control eggs receiving normal serum. By contrast, guinea pigs inoculated with similar mixtures showed no evidence of infection. In a few preliminary experiments, the addition of fresh guinea pig complement did not alter the results. It would appear that rickettsial growth in the egg resembles a tissue culture more closely than an infection in the animal body; also that the neutralization of rickettsiae is a more complex phenomenon than that of certain other disease agents. These negative experiments on neutralization indicate that this reaction requires the presence of

certain humoral or cellular elements found in adult mammals but not in the chick embryo. As Goodpasture has pointed out,¹ the chick embryo does not seem capable of acquiring immunity. In guinea pigs and mice it is possible to secure neutralization by inoculating immune serum and infectious rickettsial material at the same time, but by different routes, showing that the reaction is accomplished in the body of the animal. If we regard the embryonated egg as a tissue culture, the negative neutralization tests are not too surprising in view of the findings of Breinl²⁶ and others,^{16, 27} that the presence of immune serum in tissue cultures does not impair rickettsial multiplication. Experiments with yellow fever virus²⁸ are similar to our findings with rickettsiae, in that serum-virus mixtures that did not infect mice were still infectious for chick embryos.

Toxic Eggs.—Only yolk sacs showing very heavy rickettsial smears were used for toxin testing. As first pointed out by Gildermeister and Haagen,²⁹ yolk sacs of eggs inoculated with murine typhus may kill mice in from twenty-four to forty-eight hours after intraperitoneal inoculation. We have found, using the intravenous route, that 44 per cent of murine yolk sacs tested from eggs recently dead were lethal for mice in six hours or less at dilutions of from 1:240 to 1:2000. When intraperitoneal inoculation was done, a lower percentage of toxic yolk sacs was obtained. That epidemic typhus yolk sacs may display the same toxic property was reported by Bengtson and co-workers³⁰ who used the intravenous mode of administration. We have not succeeded in killing mice by intraperitoneal injection with this strain in the dilution employed. Of 619 yolk sacs from epidemic typhus eggs opened soon after death of the embryo and tested in mice by the intravenous route, 192, or 31 per cent, killed at dilutions of from 1:200 to 1:1200. This is a lower percentage than was found for murine yolk sacs and is no doubt due to the greater resistance of the mouse to epidemic typhus. In all probability, a larger number of toxic yolk sacs would have been obtained had living infected eggs been tested as suggested by Bengtson and co-workers, but it was usually more convenient for practical reasons to test the dead ones. We have confirmed their observation that there seemed to be no correlation between the toxicity of yolk sacs and the age of the eggs from which they were obtained. Further, the property seemed to be independent of the length of time a strain had been maintained in passage in eggs.

Efforts to demonstrate a toxin in spotted fever yolk sacs (Bitter Root strain) by mouse inoculation have been almost entirely without success. Mice were very refractory but occasionally succumbed to a 1:5 or 1:10 dilution of yolk sac administered intravenously. Better results perhaps can be obtained by lowering the resistance of the animals through various means. Experiments along these lines are in progress.

Antigen in Yolk Sacs and Yolk of Typhus-Infected Eggs.—Rickettsial suspensions are known to be unstable. We have observed that with the passage of time fewer organisms were found in smears of vaccines and antigens, although phenol or formalin was used as preservative and the materials were kept at icebox temperature. However, the value of the vaccines and antigens

was not impaired, or only slightly so, over a period of a year, as far as their immunizing or agglutinating properties were concerned. Because of the ease with which these organisms disintegrate, it is difficult to know with certainty whether antigen found in the supernatant fluid after centrifugation of rickettsial suspensions is a true soluble antigen or exotoxin, or merely a degradation product of the organisms, and perhaps an endotoxin. In this connection, Kligler and Oleinik³¹ succeeded in producing necrotic skin lesions in rabbits with typhus yolk sac suspensions both before and after high speed centrifugation, indicating that there was a toxic substance present apart from the rickettsiae. Again, one cannot be sure that this toxic substance was not a breakdown product formed during the course of preparation of the material. Castañeda was the first to observe that formalinized rickettsial bodies contained a heat-labile substance³² and also that a stable specific soluble substance could be demonstrated in rickettsial suspensions.³³ Otto and Biekhart³⁴ concluded from their experiments that the toxin produced by rickettsiae is an endotoxin, since supernatants and filtrates tested by them were nontoxic. Bengtson and co-workers³⁰ found that yolk sac suspensions which killed mice would no longer do so after the rickettsiae had been removed by centrifugation. Cohen and Chargaff,³⁵ in the first chemical study of the composition of typhus rickettsiae, found that there appeared to be two main antigenic fractions. They observed that a soluble antigen present in the supernatant of centrifuged suspensions resembled one of the fractions present in the rickettsiae themselves but did not overlook the possibility that it might have been formed by organisms which had autolyzed during cultivation. Topping and Shear³⁶ reported that a soluble substance found in the supernatants of centrifuged ether-extracted suspensions had the same immunologic properties as the resuspended sediment containing the whole organisms. We have confirmed this observation and noted in addition that the method and speed of preparation of the initial suspension are important factors and affect the quantity of soluble antigen obtained. We have found that typhus suspensions treated with ether³⁷ contain more than those prepared without this solvent and have endeavored to throw light on the question of whether the soluble antigen was separate from the organisms or merely a product of autolysis. These experiments will be reported at greater length elsewhere. Briefly, two methods of attack were employed. In one, the yolk of infected eggs was used. In the other, the effect of formalin and of ether on yolk sac suspensions of rickettsiae was studied. In all the experiments, operations were carried out with the utmost speed, and rickettsiae were separated from their supernatants as fast as possible in order to minimize the effects of autolysis.

Attempts were made to find soluble antigen in the yolks of infected eggs on the assumption that if rickettsiae produce a soluble toxin or other immunizing antigen this should be found apart from the organisms in the eggs in which they are growing. Yolk was removed from two kinds of eggs infected with murine typhus: those in which the embryo had been dead less than an hour and those of the same lot in which the embryo was still living. Mice vaccinated with the supernatants of the centrifuged yolks of both the living eggs and those recently dead showed no immunity when challenged with infectious material.

The mice receiving the resuspended sediments, which contained a very few rickettsiae, were immune. When this experiment was done with yolk from eggs that had been dead for several hours before being removed from the incubator, the supernatants immunized mice. In this case the resuspended sediments also contained more rickettsiae. We deduced from these experiments that no soluble antigen capable of immunizing was elaborated into the egg yolk by growing rickettsiae, but that when infected eggs were not removed from the incubator for several hours after death of the embryo, the yolk sacs autolyzed, liberating rickettsiae into the yolk. Disintegration of these organisms produced soluble antigen capable of immunizing mice.

Comparisons were made between 10 per cent vaccines made with and without ether, since it had been noted that ether appeared to hasten dissolution of rickettsiae. Suspensions to which 0.1 and 1 per cent formalin had been added were also compared. The large amount of formalin was tried on the theory that it would "fix" the organisms and delay disintegration. This supposition was borne out by the microscopic appearance of rickettsiae in saline suspensions one year old prepared with the two quantities of formalin. The organisms were much sharper and more distinct in the suspensions containing 1 per cent formalin. The contrast was particularly marked in ether-extracted preparations, the rickettsiae in those containing 0.1 per cent formalin being much smaller and more faded in appearance. Further, when ether-extracted vaccines were centrifuged, more soluble antigen was found in the supernatants of those containing 0.1 per cent than of those to which 1 per cent formalin had been added. Thus, in experimental studies of the antigens of rickettsiae, it would seem advisable to omit the use of ether in the starting material and to add more formalin than is customary in vaccine preparation.

SUMMARY

1. Procedures for rickettsial cultivation in fertile eggs are described that have given good results in our hands.

2. The age of chick embryos, the temperature of incubation, and the concentration of the inoculum are all important factors and are interrelated.

3. The chick embryo was found to be more susceptible to epidemic typhus and virulent spotted fever than was the guinea pig. The susceptibility of these two hosts appeared to be about the same for murine typhus.

4. No neutralization in eggs of typhus or spotted fever rickettsiae with the respective rabbit immune globulins could be obtained.

5. A higher percentage of lethal yolk sacs was found from murine than from epidemic-infected eggs when the yolk sacs were tested for toxicity by the intravenous route in mice.

6. Studies of yolk and yolk sac antigens of typhus-infected eggs indicated that the immunizing antigens found in the supernatants of centrifuged rickettsial suspensions occurred as the result of dissolution of rickettsiae during growth or manipulation.

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LIVER FUNCTIONAL IMPAIRMENT IN THERAPEUTIC MALARIA WITH PARTICULAR REFERENCE TO THE UNSUCCESSFUL USE OF METHIONINE AS A PROTECTIVE AGENT

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MALARIA inocula produces transient but readily detectable impairment of liver function. Kopp and Solomon¹ noted bromsulfalein retention, a decrease in cholesterol esters, a diminished hippuric acid excretion, and the development of a strongly positive cephalin-cholesterol flocculation test in nine patients. Evidence of liver functional derangement was likewise observed by Fredricks and Hoffbauer² in thirty-one patients receiving malarial therapy. In the latter study, the evidence of disturbed liver function consisted of mild elevations of serum bilirubin, increased excretion of urobilinogen in the urine, retention of bromsulfalein, and the development of positive cephalin-cholesterol flocculation tests. In a recent report Guttman and associates³ have clarified the mechanisms by which the cephalin-cholesterol test becomes positive in malaria. They emphasize the profound changes in the serum proteins, changes that in all probability reflect deranged hepatic function.

The development of abnormalities of liver function has been quite constant. It was evident that malaria inocula represented one form of liver injury in human beings in which a clinical study might be made of the possible effectiveness of substances which are believed to protect the liver against injury.

The sulfur containing amino acid, methionine, has been reported by Miller and Whipple⁴ to be the most effective factor in the protection of the liver against chloroform damage in protein-depleted dogs. Spontaneous liver injury develops in rats when the protein intake is inadequate, as in synthetic diets containing 10 per cent or less of casein. That the addition of methionine to such diets prevents the injury has been demonstrated by György and Goldblatt,⁵ Webster,⁶ Blumberg and McCollum,⁷ and Lillie, Daft, and Sebrell.⁸ Additional evidence for the liver protective action of this amino acid is presented in the experimental studies of Himsworth and Glynn⁹ and Goodell, Hanson, and Hawkins.¹⁰ The latter group reported some degree of protection against arsenical injury of the liver in the protein-depleted dog. There is thus ample evidence from experimental investigations that methionine aids in the protection of the liver against toxic agents in the presence of a protein-deficient diet.

The evidence for the beneficial effect of methionine when the diet prior to the injury was presumably not deficient in protein is not, however, as clear cut. Beattie and Marshall¹¹ report that the occurrence of liver damage during arsenical treatment of syphilis can be greatly reduced by the administration of methionine or casein digests. The latter are known to be a potent source of methionine. In a large series of cases of infectious hepatitis and postarsphen-

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mine jaundice, Beattie and Marshall¹² report very beneficial results by the use of methionine or of methionine containing supplements. Eddy,^{13, a, b} in two recent reports, has commented favorably on the clinical use of this substance.

When methionine administration is subjected to critical analysis, as, for example, using it for alternate cases of hepatitis, its lack of clinical value becomes apparent. Two such studies have been reported in England. Wilson, Pollock, and Harris¹⁴ studied a series of 100 cases of infective hepatitis. Alternate patients were given 5 Gm. of methionine daily. A comparison of clinical and chemical criteria revealed no significant effect. In a similar study of thirty-seven patients, Higgins and co-workers¹⁵ found that "treatment with methionine did not significantly affect the clinical course of the illness, the anorexia, or the average duration of biliuria or of bilirubinemia."

The purpose of the present study was to determine whether methionine, given during therapeutic malaria, would prevent the alterations in liver function which would otherwise be expected.*

MATERIAL AND METHODS

This study comprised a group of twelve patients admitted to the University of Minnesota Hospitals for fever therapy by inoculation malaria. All but one (Case F†) were admitted to the Dermatological Service.‡ The pertinent clinical data on these patients are shown in Table I. None of the patients had been previously subjected to malaria and none had had jaundice or infectious hepatitis in so far as could be determined by the history. In no case was there any physical evidence of hepatic disease at the time of admission (such as hepatomegaly, splenomegaly, evidence of collateral circulation, spider nevi, palmar erythema).

The malarial inoculation was carried out with citrated blood containing *Plasmodium vivax* parasites. The route was intravenous in eleven of the patients and intramuscular in one (Case F). The plan of the malarial fever therapy is to maintain a fever of over 103° F. for a total of fifty hours. This usually comprises from seven to eleven paroxysms.

All patients were offered the general hospital diet both before and during the fever. The average daily calorie intakes and the average amounts of carbohydrate, protein, and fat ingested daily during the course of the malaria are shown in Table I. All patients received fluids ad libitum. They were each given 3 Gm. of sodium chloride daily in addition to the table salt used. Alternate patients were given a supplement of 2.7 Gm. of methionine§ three times a day with meals, a total of 8.1 Gm. daily.

Under the regime described, seven of the patients lost more than three pounds in weight, the greatest loss being twenty-one pounds; five patients were able to maintain their admission weight or showed only slight loss. In the

*The possible value of such an experiment was suggested by Dr. Paul György during discussion of the previous results of Fredricks and Hoffbauer² at the Second Liver Injury Conference sponsored by the Josiah Macy, Jr., Foundation, New York, N. Y., Sept. 22, 1944.

†This case was studied on the Neuropsychiatric Service. We are indebted to Dr. J. C. McKinley for permission to make these observations.

‡We are indebted to Dr. Henry E. Mickelson of the Dermatological Service, for permission to study these cases.

§The methionine for this study was made available through the courtesy of Dr. Richard Johnson, Medical Director of John Wyeth & Brother, Inc., Philadelphia, Pa.

TABLE I. SUMMARY OF CLINICAL DATA IN THE TWELVE PATIENTS RECEIVING FEVER THERAPY BY INOCULATION MALARIA

CASE	SEX	AGE (YR.)	DIAGNOSIS	SEROLOGY		ABSENITIC INJECTIONS ESTIMATED	ORIGINAL WEIGHT (POUNDS)	AVERAGE DAILY CALORIC INTAKE	AVERAGE DAILY INTAKE			DAILY METHIONINE SUPPLEMENT (GM.)	NUMBER OF CHILLS	WEIGHT LOST (POUNDS)
				BLOOD	O.S.F.				CARBO- HYDRATE	PROTEIN	FAT			
A	F	35	Tabes dorsalis	4+	4+	2	99	700	92	27	33		4	6.5
B	M	32	Tabes dorsalis	4+	4+	32	134	1,448	170	49	64		11	5.0
C	M	38	Taboparesis	4+	4+	20	177	1,528	193	50	62		9	21.0
D	M	43	Taboparesis	4+	4+	75	136	1,454	161	59	65		12	6.0
E	F	44	Asymptomatic neuro- syphilis	4+	4+	24	123	1,133	124	46	50		10	0.0
F	F	46	Taboparesis	4+	4+	1	131						9	2.0
G	M	26	Congenital general paresis	4+	4+	90	156	974	148	37	39	8.1	5	3.0
H	M	40	Latent syphilis	4+	Negative	2	159	990	112	42	49	8.1	7	7.0
I	M	36	Early paresis	4+	4+	24	166	1,444	169	55	61	8.1	11	10.0
J	M	44	Asymptomatic neuro- syphilis	4+	4+	27	188	2,105	244	86	81	8.1	8	0.0
K	F	44	Early paresis	4+	4+	0	145	985	102	39	47	8.1	9	6.0
L	M	34	Tabes dorsalis	4+	4+	42	139	1,083	131	41	45	8.1	11	3.0

We are indebted to the hospital dietitians, Miss Gertrude Thomas and her associates, Misses C. Weaver and S. Adams, for recording and calculating the caloric intake and the composition of the daily food intake.

twelve patients, the average number of chills was 8.8 and the average number of hours of fever over 103° was forty-two.

Four laboratory methods of detecting disturbed liver function were used. They were selected on the basis of sensitivity and simplicity. Determinations were carried out on three occasions in each instance: (1) prior to the development of fever, (2) approximately halfway through the course of the malarial paroxysms, (3) from one to three days after the termination of fever by quinine.

The level of the serum bilirubin was measured by the modification of the Malloy and Evelyn technique¹⁶ recently published by Ducci and Watson.¹⁷ The one-minute or prompt direct-reacting bilirubin rarely, if ever, exceeds 0.2 mg. per 100 c.c. under normal circumstances.* The total bilirubin, measured after additions of alcohol, is usually less than 1.0 mg. per 100 c.c. Elevations of the delayed- and indirect-reacting bilirubin (total minus one minute or T - 1-minute fraction) are characteristic of retention jaundice, while elevations of the one-minute or prompt direct-reacting bilirubin are seen in regurgitation jaundice, whether due to parenchymal liver disease or extra hepatic biliary obstruction.

The bromsulfalein test is a helpful means of recognizing impaired liver function in the nonjaundiced patient. The technique advocated by Mateer, and co-workers¹⁸ was employed in the present study. Five milligrams of the dye per kilogram of body weight were given intravenously in the fasting state. A single sample of blood was withdrawn at forty-five minutes. The determinations were made on the serum after protein precipitation. The concentration of the retained dye was determined in the Evelyn photoelectric colorimeter; a concentration of 10 mg. per 100 c.c. was considered to be 100 per cent. This is based on the arbitrary assumption of a plasma volume of 50 c.c. per kilogram as explained by Lichtman.¹⁹ Normal individuals show no retention of the dye at forty-five minutes. The use of the photoelectric colorimeter permits a more sensitive and accurate reading than can be obtained with the comparator block.

The excretion of increased amounts of urobilinogen in the urine is believed to be an evidence of reduced hepatocellular function. The method of estimating the intensity of the Ehrlich reaction²⁰ was employed. This is not a precise method of determining urobilinogen, but it reveals increases of the latter substance with sufficient accuracy and faithfulness for clinical purposes. The amount of Ehrlich-reacting substance is recorded in Ehrlich "units" for a two-hour urine sample from (2 to 4 P.M.). Ninety-five per cent of normal individuals between the ages of 18 and 25 years were found to excrete less than 1 unit in the two-hour sample. While the exact upper limit of normal for all ages and conditions, such as exertion, fatigue, and type of diet, cannot be stated, it is believed that values above 1.5 are in all probability abnormal.* In most instances in the present study, analyses were made on samples from 2 to 4 P.M. collected on two successive days during each of the three periods.

The development of a positive cephalin-cholesterol flocculation reaction in parenchymal hepatic disease as observed initially by Hanger²¹ is well known. The occurrence of a positive test in malaria has been noted by Kopp and Solomon,¹ by Greene,²² and by Mirsky and associates.²³ The studies of Guttman and

*Personal communications from Dr. C. J. Watson.

co-workers³ demonstrate clearly the relationship of this phenomenon to alterations in the serum proteins induced by malaria. In the study of Fredricks and Hoffbauer,² referred to previously, twenty-two of twenty-eight patients exhibited 3 plus or 4 plus reactions immediately after completion of malarial therapy. The values in that study were reported as forty-eight-hour readings. In the present group of cases, the values are reported as the twenty-four-hour reading; lower values, 2 plus and 3 plus reactions, have greater significance at twenty-four than at forty-eight hours.

RESULTS

In Figs. 1 to 4 are illustrated the results of the liver function tests before, during, and after malaria therapy in the control patients and in the alternate patients with methionine added to their diets.

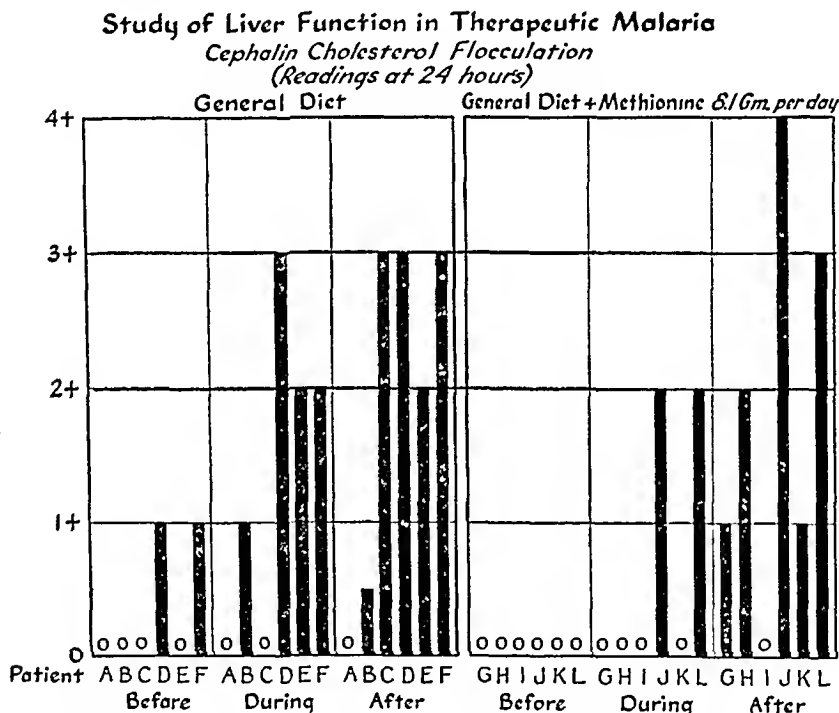


Fig. 1.—Cephalin-cholesterol flocculation test in twelve patients receiving induced malarial fevers.

The cephalin-cholesterol flocculation test is shown in Fig. 1. It is apparent that there is no appreciable difference between the control and methionine-fed groups. It was observed that the test tended to become positive rather late in the course of the malaria and remained positive after the malaria had been terminated.

The results of the one-minute and total serum bilirubin determinations are shown in Fig. 2. It is seen that the total serum bilirubin was elevated during malaria in seven patients and within normal limits in five. Of these

five, one was in the control group and four were in the methionine-fed group. One patient (Case C) showed a uniformly high serum bilirubin throughout the period of study. This patient undoubtedly had a rather pure retention jaundice, probably of hemolytic type, since the mean cell diameter of the red blood cells was but 6.8μ , and the fragility was moderately increased (H_1 0.50, H_2 0.32; control, H_1 0.44, H_2 0.32). Unfortunately, the feces urobilinogen was not determined. It is of interest to note that the level of the total serum bilirubin in this patient actually decreased slightly during the febrile period, although there was a slight increase in the concentration of the one-minute bilirubin.

Study of Liver Function in Therapeutic Malaria

Serum Bilirubin

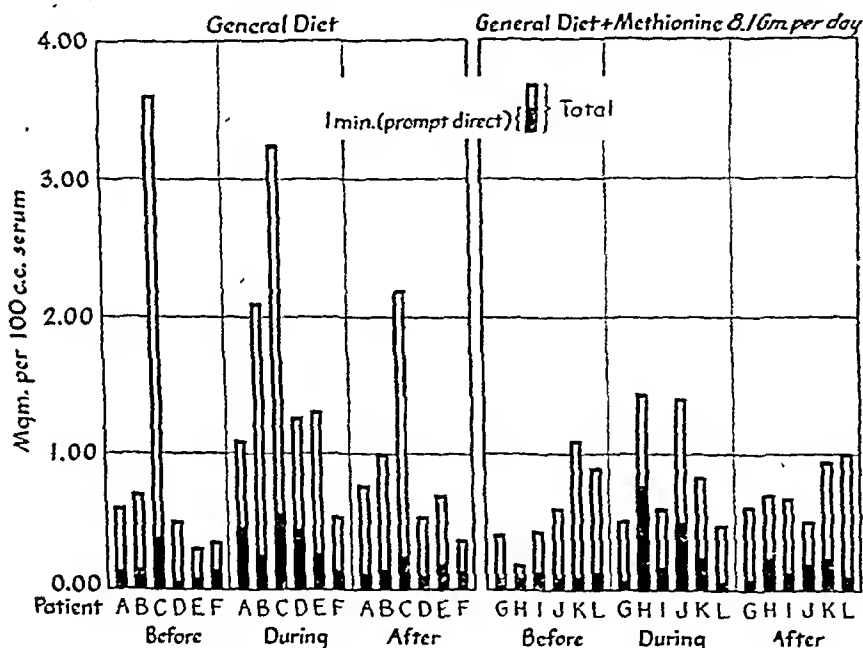


Fig. 2.—The quantitative serum bilirubin determination in twelve patients receiving induced malarial fever. The upper limit of the one-minute value in the normal is accepted as 0.2 mg. per cent and the total as 1.0 mg. per cent. For discussion of this and of the abnormal initial value in Case C, see text.

This decrease is somewhat difficult to reconcile with the usual explanation of retention jaundice, namely, diminished hepatocellular ability to clear the blood bilirubin. There was, in all probability, an actual increase in production of bilirubin during this period because of the increased rate of blood destruction which is so characteristic of malaria. Despite the increased load, the liver appeared to have actually "cleared" the bilirubin more effectively, if one can judge from the values noted. The patient was studied six months later and again the total serum bilirubin was elevated, 2.6 mg. per 100 c.c., although the one-minute reading was only 0.2 mg. per 100 c.c. The liver and spleen at this time were enlarged and readily palpable. As already noted, the fragility of the erythrocytes was somewhat increased. The red blood cell count and the hemoglobin values were normal. The number of circulating reticulocytes was

Study of Liver Function in Therapeutic Malaria

Quantitative Ehrlich Reaction in 2 to 4 PM Urine Specimen
(Examinations on two successive days)

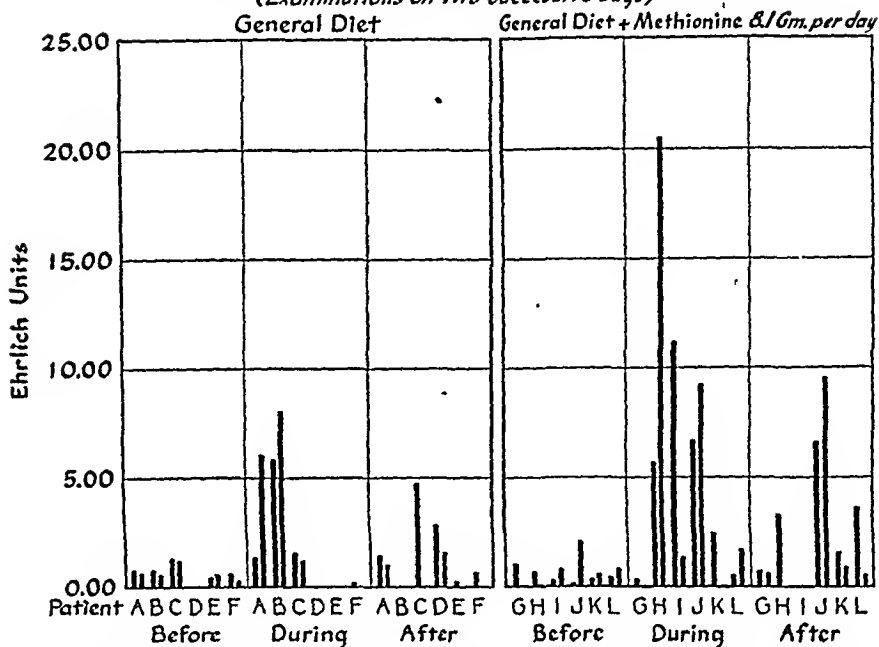


Fig. 3.—The quantitative urine Ehrlich reaction, a measure of urobilinogen excretion, in twelve patients receiving induced malarial fevers. The upper limit of normal by this method is 1.5 units.

Study of Liver Function in Therapeutic Malaria

Bromsulphthalein

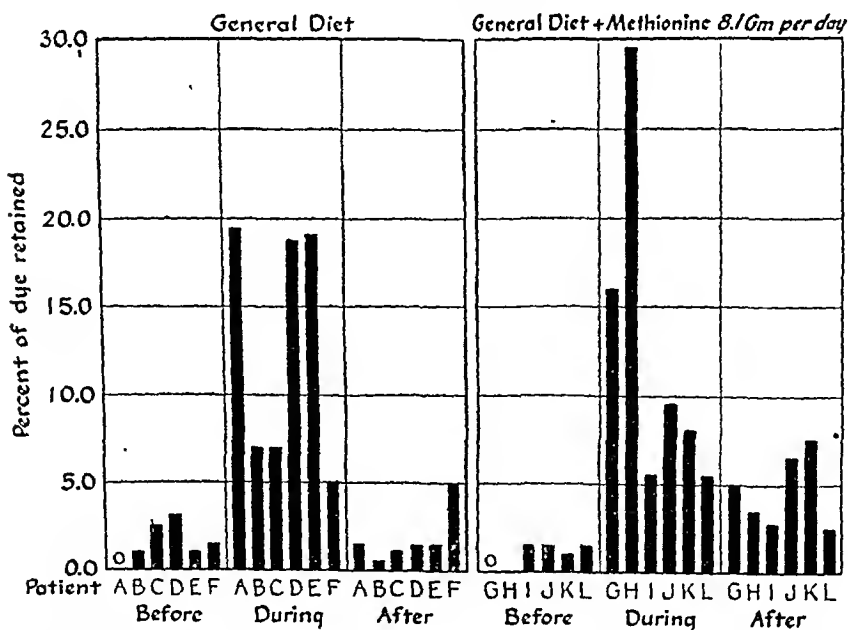


Fig. 4.—Degree of bromsulphthalein retention in twelve patients receiving induced malarial fevers. Readings were made forty-five minutes after the injection of 5.0 mg. of the dye per kilogram of body weight.

normal. Thus the evidence, although equivocal, suggests that the case is more likely an example of latent familial hemolytic jaundice rather than constitutional hyperbilirubinemia.

The Ehrlich reaction on 2 to 4 P.M. urine samples on two consecutive days before, during, and after malaria is shown in Fig. 3. This value was elevated in nine of eleven patients in whom the test was performed during malaria and in seven of eleven patients in whom the test was performed following termination of the malaria. No appreciable difference was noted between the results in the control patients and those given methionine.

The results of the bromsulfalein test are shown in Fig. 4. Positive results are noted in every instance during malaria. In one instance (Case H) the initial test was not run until the patient had already had seven and one-half hours of fever over 100° F.; 19 per cent retention was observed. At the same time, in this instance, all the other tests were still within normal limits. Later, as is shown in Fig. 4, the degree of dye retention in this patient (Case H) was still greater (29 per cent). Again there was no appreciable difference between the control and the methionine-fed groups as judged by the bromsulfalein test.

DISCUSSION

Impairment of liver function, detectable by suitable laboratory tests, has again been observed in patients receiving therapeutic malaria. These patients have been studied in order to evaluate the possible protective effects of extra methionine over and above that contained in the diet. Such a method of clinical evaluation seemed especially desirable in view of some of the favorable clinical reports¹¹⁻¹³ on the beneficial effects of methionine in hepatic disorders. In six patients with malaria, methionine, given orally in doses of 8.1 Gm. daily, exerted no beneficial effects that could be detected. The methionine-treated patients exhibited the same abnormalities as did those in the control group. The disturbances in liver function are usually transitory and revert to normal sometime after termination of the fevers.

SUMMARY

1. Twelve patients with therapeutic malaria were studied before, during, and after the malaria with regard to liver function. Four laboratory tests, the cephalin-cholesterol flocculation, the quantitative serum bilirubin, the quantitative Ehrlich reaction, and the bromsulfalein test, were used.
2. Alternate patients, six of the twelve, were given 8.1 Gm. of methionine orally per day in addition to a general hospital diet. The other six served as controls and received the same diet without extra methionine.
3. All twelve of the patients studied exhibited some evidence of disturbed hepatic function as measured by the four tests.
4. In ten of the twelve patients, there were functional abnormalities from one to three days after the termination of the malaria.
5. The daily addition of methionine to the diet failed to provide any evidence of protection of liver function.

The authors wish to express their appreciation to Dr. C. J. Watson for his encouragement and helpful suggestions in this study.

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THE FORMATION AND EXCRETION OF ACETYLATED SULFONAMIDES

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WHILE a fairly comprehensive literature on the pharmacology of the principal sulfonamides exists, the systematic investigation of their acetylated derivatives has been neglected, comparatively, except in a few instances. However, we have felt that a fairly comprehensive study of their rates of acetylation, the plasma protein binding of the acetylated forms, their renal clearance and the effect of alkali thereon, and the rate of falling plasma concentration of these conjugated sulfonamides would yield information of considerable benefit to the over-all understanding of the pharmacology of these agents. The acetylated forms of sulfamerazine, sulfadiazine, sulfamethazine, and sulfathiazole have been the compounds used in this investigation.

EXPERIMENTAL

Study of the Rate of Acetylation.—A study of the rate of acetylation of sulfamerazine, sulfadiazine, sulfamethazine, and sulfathiazole was carried out in two-stage bilaterally nephrectomized rats. This operative measure was considered desirable since the rate of acetylation and the clearance of both the free and combined forms of the compounds varied considerably. Actually, curves presented in the literature representing the amount of conjugated sulfonamide appearing in the blood stream are integrations of the operation of these several factors.

Albino rats weighing between 200 and 300 grams were nephrectomized unilaterally and allowed ten days or longer in which to recover from this first stage. Following recovery the second kidney was removed late in an afternoon and the animals were allowed to recover overnight from the surgical procedures. This tended to minimize the shock of the operations, to yield a low mortality, and to produce animals which lived for about ninety-six hours.

The morning following surgery the rats were injected intraperitoneally with 50 mg. per kilogram of one of the sulfonamides. Free and total sulfonamide determinations¹ were made on samples of whole blood obtained two, four, six, and twenty-four hours after administration of the compounds. Thus the experiments were completed while the animals could be considered reasonably normal.

The results are presented in Fig. 1. The curves for any one drug represent the average percentage acetylation using approximately seventeen animals.

¹From the Department of Pharmacology, The Medical Research Division, Sharp and Dohme, Inc.

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Most notable is the very rapid and extensive acetylation of sulfathiazole; sulfamerazine is conjugated slowest and to the least extent. Of the sulfonamide present in the blood stream twenty-four hours following administration, the percentage of each conjugated was 69.4 for sulfathiazole, 47.7 for sulfamethazine, 42.9 for sulfadiazine, and 35.7 for sulfamerazine. However, it is possible that these absolute values are not as reliable as the early rates of conjugation, if it be acknowledged that over a long period of time the free and combined compounds could be excreted into the gastrointestinal tract or otherwise eliminated at different rates.

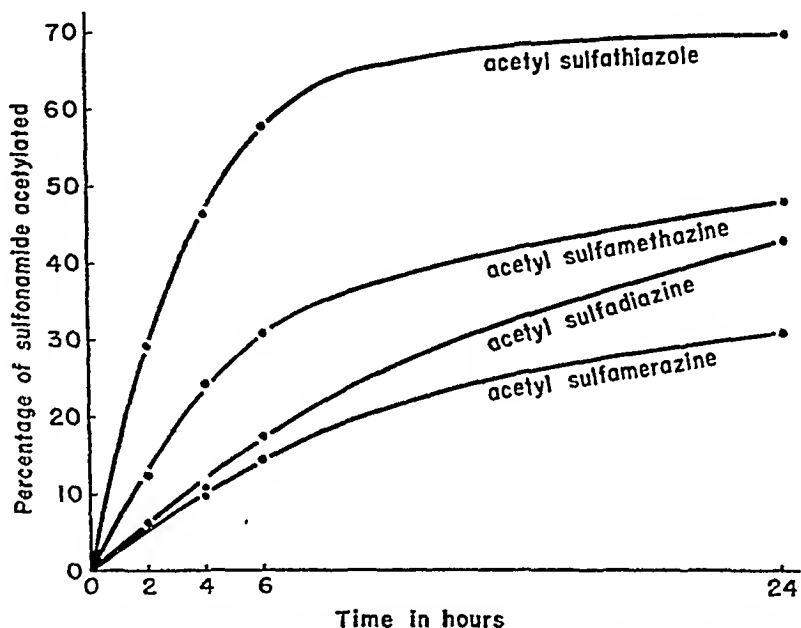


Fig. 1.—Demonstrating the percentage acetylation of sulfonamides in bilaterally nephrectomized rats as calculated from their free and combined concentration in blood.

As a check on these results, we repeated the experiments using normal rats and the same dosage of sulfonamides. Since the blood levels fell off rapidly, and the differences between free and total sulfonamide were not great, these data have been omitted from Fig. 1. The absolute values for these are included in Table I. The principal difference between the two sets of data is that in the latter experiments the percentage of combined sulfamerazine in the blood stream was slightly greater throughout than that of combined sulfadiazine. This is in accordance with the findings of Welch and associates.²

The data on the bilaterally nephrectomized animals, especially the twenty-four hour values, while not complicated by urinary excretion, do not account for the gradual falling off of both free and total sulfonamide content of the blood (Table I), which is probably due to excretion directly into the gastrointestinal tract, indirectly through the hepatobiliary system, or inactivation in some other manner.

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TABLE I. COMPARISON OF FALLING PLASMA CONCENTRATION OF FREE AND TOTAL SULFONAMIDES IN NORMAL AND BILATERALLY NEPHRECTOMIZED RATS FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 50 MG. PER KILOGRAM (DATA REPRESENTS AVERAGE FIGURES [MILLIGRAMS PER 100 C.C.] FOR FROM TWELVE TO SEVENTEEN ANIMALS FOR EACH EXPERIMENT)

HR.	SULFAMERAZINE				SULFAMETHAZINE				SULFADIAZINE				SULFATHIAZOLE	
	NEPHRECTOMIZED		NORMAL		NEPHRECTOMIZED		NORMAL		NEPHRECTOMIZED		NORMAL		NEPHRECTOMIZED	
	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG	FREE DRUG	TOTAL DRUG
2	8.0	8.5	7.9	8.2	6.5	7.4	7.5	8.2	8.4	8.9	6.9	7.1	3.8	5.3
4	7.3	8.1	6.0	6.3	5.6	7.4	5.5	6.1	7.9	8.9	5.2	5.3	2.8	5.2
6	6.5	7.5	5.2	5.5	4.6	6.6	4.0	4.4	7.0	8.4	4.2	4.1	2.1	4.8
24	3.4	5.3	1.0	1.2	2.0	4.0	0.2	0.5	3.8	6.8	0.4	0.7	0.8	2.6

Since in these experiments we eliminated the renal factor in the pharmacodynamic picture, the renal clearance of the compounds was evaluated as a separate study.

Renal Clearances of Acetylated Sulfonamides.—The renal clearances of acetylated sulfonamides were investigated in dogs in a manner strictly comparable to the procedure which we previously described.³ Briefly, simultaneous sulfonamide and creatinine clearances were performed before and after the oral administrations of 7 Gm. of sodium bicarbonate, or additional water. The bicarbonate or water was added to alkalize the urine or induce diuresis, the two common procedures for decreasing the tendency of sulfonamides to produce crystalluria. The amount of sulfonamide administered subcutaneously at the beginning of each experiment was 0.5 Gm.

TABLE II. SUMMARY OF DATA ON RENAL CLEARANCE OF ACETYLATED SULFONAMIDES NORMALLY AND AS INFLUENCED BY ALKALIZATION AND DIURESIS

SULFONAMIDE	ADMIN. SOD. BICARB. OR ADD. WATER*	PLASMA CONCENTRATION (MG./100 C.C.)	URINE (VOL./MIN.)	CREATININE CLEARANCE	SULFONAMIDE CLEARANCE†	CLEARANCE RATIO	pH
<i>Effect of NaHCO₃ (pH)</i>							
Acetylsulfamerazine	B	4.6	4.26	59.2	58.4	0.99	6.25
	A	3.9	4.74	62.7	71.3	1.23	7.45
Acetylsulfamethazine	B	5.8	3.61	64.0	44.4	0.70	6.41
	A	6.5	4.58	54.8	50.7	0.95	6.93
Acetylsulfadiazine	B	4.4	4.32	63.3	56.6	0.90	6.38
	A	4.1	3.38	57.5	62.3	1.08	7.97
Acetylsulfathiazole	B	5.5	4.46	69.7	122.8	1.78	6.49
	A	4.4	3.35	65.1	176.5	2.71	8.03
<i>Effect on Low and High Urine Flow</i>							
Acetylsulfamerazine	B	4.4	0.51	68.8	59.6	0.88	5.80
	A	4.1	5.30	70.1	79.6	1.12	6.40
Acetylsulfamethazine	B	6.6	0.50	54.7	34.4	0.61	6.84
	A	6.4	4.05	61.8	45.5	0.72	6.50
Acetylsulfadiazine	B	4.6	0.64	56.1	50.8	0.88	6.53
	A	3.4	4.67	60.8	62.8	1.02	6.49
Acetylsulfathiazole	B	5.1	0.42	61.5	92.3	1.51	7.59
	A	4.1	4.32	63.0	157.6	2.51	6.52

*B, Before; A, After.

†Corrected for binding on plasma albumin.

The data averaged in Table II demonstrate much higher renal clearances of the acetylated compounds than of the corresponding free drugs. The average normal clearances in cubic centimeters per minute were acetylsulfamerazine, 58.4; acetylsulfamethazine, 44.4; acetylsulfadiazine, 56.6; and acetylsulfathiazole, 122.8. The clearances of the corresponding unacetylated amines were sulfamerazine, 9.3; sulfamethazine, 7.1; sulfadiazine, 15.8; and sulfathiazole, 35.4.³

While either alkalization or increasing urine flow increased the clearances of the acetylsulfonamides, their effects were not as pronounced on a percentage basis as were their effects on the unconjugated compounds. However, the fact that both these measures increased the clearances of the compounds, even where their normal clearance ratios were about or above 1.0, made it appear that they were reabsorbed passively to some extent and that this property was influenced considerably by the concentration of electrolyte in the urine. This was especially notable in the case of acetylsulfathiazole.

There are several reports that indicate that the acetylated forms of those sulfonamides tested are secreted by the renal tubular epithelium of man. Thus, Earle⁴ found the average clearance ratio for sulfamerazine to be 0.15, whereas the corresponding value for acetylated sulfamerazine was 2.42. Parenthetically, a value for clearance ratio less than 1.0 indicates active or passive reabsorption; a value greater than 1.0 is taken to indicate tubular excretion of a compound. Loomis and co-workers⁵ reported the clearance ratio of sulfanilamide to be 0.45 and that for acetylsulfanilamide to be 1.03. These data are not corrected for binding of the sulfonamide on plasma protein and hence are not strictly comparable to Earle's results, although such a correction would not influence these data qualitatively or to a great extent quantitatively, judging from the reported figures for the plasma binding of sulfanilamide⁶ and from what is reported herein about the comparative binding of free and acetylated sulfonamides. Reinhold and associates⁷ found the clearance ratios of free and acetyl sulfadiazine to be 0.31 and 0.82; sulfathiazole, 0.87 and 2.11; and, interestingly enough, sulfapyridine, 0.28 and 0.23. On the whole, it appears that both man and dogs tend to reabsorb and so retain the therapeutic form of the drugs and to excrete the inactive form of the agents at a relatively very rapid rate. By contrast, penicillin, the principal representative of another class of chemotherapeutic agents, has the very highest renal clearance. It is cleared from the blood stream as rapidly as it is brought to the nephron.⁸

Binding of Acetylated Sulfonamides on Plasma Proteins.—The binding of acetylated sulfonamides on plasma proteins is, in general, of much the same order of magnitude as that of the unacetylated or free compounds. This generalization is borne out by the data presented in Table III. There are two discrepancies in the table worthy of note and speculation as to cause. In every instance the binding of these compounds on human plasma proteins is greater than on dog plasma. The method was the same throughout, being essentially the equilibration of sulfonamide in buffer and plasma by dialysis through a Visking membrane and correcting the results to a standard plasma albumin concentration. Also, there appears to be very good agreement between the data

TABLE III. COMPARISON OF BINDING OF FREE AND ACETYLATED SULFONAMIDE ON HUMAN AND DOG PLASMA: THE INITIAL EQUILIBRIUM CONCENTRATION ON BOTH SIDES OF THE MEMBRANE, 5 MG. PER 100 C.C.; TEMPERATURE, 37° C.; DURATION, EIGHTEEN HOURS (RESULTS CORRECTED TO A PLASMA ALBUMIN CONCENTRATION OF 4 GM. PER 100 C.C. FOR MAN AND 3.5 GM. PER 100 C.C. FOR DOGS)

COMPOUND	PERCENTAGE BOUND	
	HUMAN PLASMA	DOG PLASMA
Sulfamerazine	78.2	36.4
Acetylsulfamerazine	79.7	44.6
Sulfamethazine	89.2	60.7
Acetylsulfamethazine	90.4	64.6
Sulfadiazine	44.8	16.4
Acetylsulfadiazine	49.1	28.7
Sulfathiazole	70.9	53.2
Acetylsulfathiazole	75.5	79.8

for free and acetylated compounds in human plasma and somewhat more freedom in the comparisons based on dog plasma protein binding.

Davis has reported,⁹ and we have confirmed his finding, that essentially all the binding of sulfonamides is on the albumin fraction of plasma, but we have seen that the binding of these same agents varies definitely from one species to another. A most interesting observation of Reinhold and Flippin¹⁰ which may well fit into this discussion was that the binding of sulfonamides on the plasma of patients with cirrhosis or those in the late stages of certain infectious diseases was considerably less than normal, although the values were corrected to standard plasma albumin concentration. Thus, it appears that not only is the amount of albumin altered in these diseases, but also its binding characteristics. One might speculate that this difference in albumin binding of compounds due to disease or species difference may be as important as the role of that protein in hemodynamics.

Plasma binding experiments were performed over a range of concentrations from 5 to 100 mg. per 100 c.c. for both the free and acetylated compounds. These were for the purpose of determining whether the compounds followed the conventional Freundlich isotherm over this range of concentrations. In Fig. 2

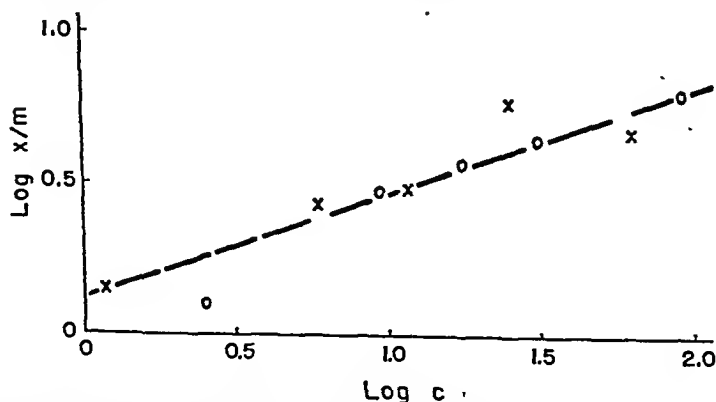


Fig. 2.—Plasma protein binding of sulfamerazine, O, and acetylsulfamerazine, X, expressed as an isotherm. Temperature and duration of equilibration were 37° C. and eighteen hours, respectively; pH was buffered at 7.4.

is illustrated the "fit" of such binding data for a single sulfamerazine experiment to these conditions as being representative of the group. It is also apparent that both the unconjugated and acetylated compounds may be represented by the same curve.

Falling Plasma Concentrations of Free and Acetylated Sulfonamides.—The falling plasma concentrations of free and acetylated sulfonamides can be compared easily in dogs by administering either form of a compound with the assurance that the free form will not be conjugated. On the whole, then, the rate of fall of these plasma concentrations, when the individual sulfonamides are administered subcutaneously, is substantially a function of their renal clearances.

TABLE IV. COMPARISON OF PLASMA CONCENTRATIONS OF FREE AND ACETYLATED SULFONAMIDES FOLLOWING SUBCUTANEOUS ADMINISTRATION OF 1 GM. OF DRUG TO DOGS

	AVERAGE PLASMA CONCENTRATION IN MG./100 C.C.					
	1 HR.	2 HR.	4 HR.	6 HR.	12 HR.	24 HR.
Sulfamerazine	8.3	8.7	7.6	6.3	3.9	1.7
Acetylsulfamerazine	7.8	6.9	4.5	3.4	1.4	0.7
Sulfadiazine	7.5	7.8	6.8	5.4	2.2	1.1
Acetylsulfadiazine	6.4	6.4	5.2	3.7	1.5	0.3
Sulfamethazine	8.4	8.7	7.0	5.5	3.3	1.3
Acetylsulfamethazine	8.8	7.6	6.0	4.2	1.7	0.5
Sulfathiazole	5.5	6.1	4.8	3.1	1.2	0.3
Acetylsulfathiazole	7.2	6.3	4.0	2.4	0.4	0.1

It may be seen, by comparing the data in Table IV, that the fall of the plasma concentration of all the acetylated compounds was much more rapid than that of the free form of the drugs. These data represent averages of eleven experiments on seven dogs for the free compounds and four experiments on four dogs for the conjugated sulfonamides. Four of the animals were used for both types of experiments. One gram of the drug was administered subcutaneously to each animal.

SUMMARY

This work has served to bring together in a single study a number of the principal problems having to do with the formation and elimination of acetylated sulfonamides. The results may be summarized in a general manner as follows:

1. Sulfathiazole is conjugated in the body at a considerably greater rate and to a greater extent than are the pyrimidine derivatives. Of the latter group, sulfamethazine is conjugated most rapidly and to the greatest extent.

2. At a given plasma concentration, both the free and conjugated sulfonamides are bound to approximately the same extent on plasma proteins. This is especially true for human plasma albumin and less true for dog plasma, which normally binds sulfonamides to a lesser extent.

3. The renal clearances of all the acetylated sulfonamides tested were greater than those of the unconjugated form, the clearance of acetylsulfathiazole being by far the greatest. Even though the clearances of the compounds, except for acetylsulfamethazine, approximated or exceeded the glomerular filtration, sodium bicarbonate or the production of diuresis increased still further the

clearances of all the sulfonamides. Thus, these measures for the minimizing of sulfonamide renal complications appear to impede the probably passive reabsorption of the compounds.

4. It follows from a consideration of results 2 and 3 that the plasma concentration of the acetylated forms of these agents should fall more rapidly after a single standard parenterally administered dose than after the injection of the unconjugated form of the agents. This was found to be true.

5. Finally, any attempt to integrate all these variables together with those for the unconjugated sulfonamides immediately becomes very involved when expressed in writing for even a single compound. However, an intimate working knowledge of them is requisite to a full appreciation of the advantages and limitation of present compounds and is of uppermost importance in the pharmacologic evaluation of new chemotherapeutic agents.

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MISCELLANEOUS PHARMACOLOGIC ACTIONS OF CITRININ

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CITRININ is the antibiotic substance produced by the molds *Penicillium citrinum* Thom,¹ *Aspergillus terreus* Thom,² and *Aspergillus candidus*.³ It has been also found in a flowering plant *Crotolaria crispata*.⁴ Its chemical composition and structure have been established chiefly by Hetherington and Raistrick¹ and Coyne, Raistrick, and Robinson.⁵ It is an organic acid but has not been synthesized. It is stable and can be autoclaved at fifteen pounds of pressure for twenty minutes without loss of antibiotic power.⁶ It has a fairly high antibiotic action on most gram-positive bacteria,⁷⁻⁹ but, unfortunately, it is rather toxic for higher animals.¹⁰

According to Timonin,⁶ citrinin is useful, though weaker than penicillin, in the local treatment of sore throat, of the common cold, and of abscesses. However, nothing is known about its local effects, possible injuriousness to mucous membranes and skin, and systemic effects and absorption from various regions, especially from local applications. This report presents results of various tests aimed to provide this desirable information.*

LOCAL EFFECTS ON SKIN AND MUCOUS MEMBRANES

Skin.—On human and rabbit skin, citrinin in the form of pure powder, ointments of from 1 to 5 per cent, and aqueous solutions of from 1 to 20 per cent made from its sodium salt produced no demonstrable irritation. When injected intradermally in man, the sodium citrinin in 1 and 2 per cent strengths also produced no demonstrable effects. When injected intradermally in rabbit skin or subcutaneously, it produced only slight redness, which was stained locally a slightly bluish color after intravenous injection of trypan blue, 10 mg. per kilogram, thus suggesting slight capillary dilatation.

Mucous Membranes.—When the nose and pharynx of four persons were sprayed by atomizer with citrinin sodium 0.05 per cent (pH 7.0), there was no, or barely detectable, irritation; 0.1 per cent caused definite, though moderate, burning, and 0.2 per cent still more. Spraying, instillation of drops, or topical applications with cotton pledgets of solutions of 1 and 2 per cent produced an immediate and marked burning sensation with lachrimation which lasted for about ten minutes. Instilled into conjunctivas of three rabbits, a 1 per cent solution produced no effects; 2 per cent, a moderate hyperemia, but no chemosis; 5 per cent, chemosis with profuse lachrimation. One-tenth per cent solution produced no demonstrable effects on the conjunctivas of two cats, but 1 per cent produced hyperemia, and 2 per cent, chemosis and lachrimation. On the frog web, 0.6 per cent solution produced a slight vasodilatation and 0.8 per cent, a

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marked and persistent vasodilatation. Generally, for mucous membranes, therefore, citrinin is somewhat irritating in concentrations of 0.1 and 0.2 per cent but not objectionable in concentrations of 50 mg. per cent used as spray; much higher concentrations are definitely and sometimes markedly irritating.

Ciliary Activity.—Isolated frog esophagus was split into halves longitudinally, spread out flat, and the time required for a small granule of cork to travel a distance of 1 cm. was recorded. An average of three successive results was used as the state of initial activity. Then a cotton pledget soaked with Ringer's solution was applied to one-half the esophagus (control), a pledget soaked with citrinin sodium solution (pH 7.0) to the other half, each for fifteen minutes, the ciliary activity redetermined, and any differences estimated.

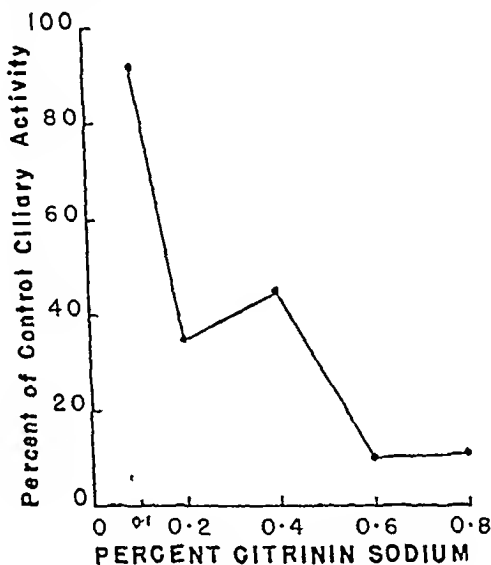


Fig. 1.—Effect of citrinin sodium in different concentrations on ciliary activity in frog esophagus.

The results on three frogs used for each concentration of citrinin were as follows: 0.1 per cent solution produced only a slight slowing of the ciliary activity; 0.2 to 0.4 per cent depressed it to about one-third to one-half and 0.6 to 0.8 per cent to about one-tenth the original activity. Sometimes the highest concentrations caused stoppage of the cilia. However, the strongest depression was completely reversible by washing with saline solution, when washed within twenty minutes. The results are presented graphically in Fig. 1.

NERVE FUNCTIONS

Reflex Time.—The reflex time was determined by dipping the legs of decapitated frogs into 0.5 per cent hydrochloric acid according to the Tuerek method. The average of three results gave the initial reflex time. Then one foot was soaked in Ringer's solution (control) and another in citrinin sodium solution, each for fifteen minutes, the reflex time redetermined, and any difference estimated. The results on three frogs for each solution of citrinin sodium from 0.1 to 0.6 per cent produced no demonstrable effects on the reflex time. With

concentrations of 0.8 to 2 per cent the reflex time was doubled, thus indicating some depression of sensory nerves. However, complete local anesthesia was not produced.

Contralateral Reflex Time.—The sciatic plexuses of decapitated frogs were exposed and contralateral reflex time for legs was determined by electrical stimulation of an opposite leg, an average result being obtained from three successive determinations. Then one plexus was covered with cotton soaked in Ringer's solution (control) and the opposite plexus with cotton soaked in a citrinin sodium solution, each for fifteen minutes, when the contralateral reflex times were redetermined. The average of three frogs for solutions of citrinin sodium from 0.1 to 0.8 per cent showed no differences from the controls, thus indicating that sensory conduction in the fibers was not affected.

Motor Nerve Irritability.—Threshold faradic stimuli were determined for the sciatic nerve trunks of frog muscle-nerve preparations. The stimulations were made before and after wrapping the nerves with cotton pledgets soaked in Ringer's solution (control) and their companions with pledgets soaked in citrinin sodium in concentrations from 0.1 to 0.8 per cent, each for fifteen minutes, when the thresholds were redetermined. The averages for three frogs used for each solution showed that there were no demonstrable changes in sciatic nerve irritability.

ABSORPTION FROM MUCOUS MEMBRANES

Nose.—Citrinin sodium in 20 per cent solution was instilled in doses of from 25 to 120 mg. of citrinin per kilogram, using 0.1 c.c. of solution every five minutes, into the noses of four rabbits, unanesthetized, and two cats and one dog anesthetized with pentobarbital (30 mg. per kilogram intraperitoneally), all animals being tied down on their backs with heads lower than their bodies. However, the presence of citrinin in the blood and urine was questionable or practically negligible (Table I). The solutions were left in the noses for from one-half to two hours. The citrinin was estimated by Wolohan and Cutting's capillary tube system¹¹ using *Staphylococcus aureus*, the sensitivity limit to citrinin being an average of 1:16,000, and *Streptococcus hemolyticus*, the sensitivity limit being an average of 1:40,000 (Table II).

In two of the cats, the carotid blood pressure was recorded, but no changes were demonstrable from the nasal instillations. However, control intravenous injections of 10 mg. per kilogram of citrinin in the same animals caused a prompt fall in blood pressure. Similar nasal instillations of 0.2 c.c. 20 per cent mecholyl (0.4 Gm. total) in the same cats caused a prompt fall in blood pressure, thus showing that the nasal region was functionally capable of absorption.

Alimentary Canal.—Introduction of the same citrinin solution in the same dosage (as in the nose) into the mouths of two similarly pentobarbitalized cats with the esophagus tied at the neck resulted again in questionable or negligible amounts in the blood and urine (Table I). Injection into ligated esophagus and stomach, and loops of duodenum, ileum, and colon of twenty-six similarly pentobarbitalized rats, doses of from 25 to 50 mg. per kilogram, resulted in definite absorption from all these organs. Blood levels from all organs of from 1:4,000 to 1:32,000 (average about 1:15,000) could be detected and of about the

TABLE I. ABSORPTION OF CITRININ SODIUM FROM DIFFERENT REGIONS AND ORGANS

CITRININ SODIUM (MG./KG.)	ORGAN OR REGION	ANIMAL	AVERAGE CONCENTRATION OF CITRININ	
			BLOOD†	URINE†
50	Esophagus	Rat	1:16,000	
25	Esophagus	Rat (4)*	1:10,000	
50	Stomach	Rat (4)*	1:9,500 (1:4000 to 1:16,000)‡	
25	Stomach	Rat	<1:20,000	
50	Duodenum	Rat (4)*	1:12,000 (1:4000 to 1:20,000)‡	
25	Duodenum	Rat	<1:20,000	
50	Ileum	Rat (3)*	1:15,000 (1:400 to 1:32,000)‡	
50	Colon	Rat (4)*	1:9,500 (1:4000 to 1:16,000)‡	
25	Colon	Rat	1:20,000	
25	Nose	Dog	<1:16,000 (S)	<1:16,000
25	Nose	Cat	<1:40,000 (H)	<1:40,000 (H)
50	Nose	Rabbit (3)*	<1:16,000 (S)	<1:16,000
50	Nose	Cat	<1:32,000 (H)	<1:32,000 (H)
25	Mouth	Cat (2)*	<1:40,000 (H)	<1:40,000 (H)
50	Intramuscular	Rat (4)*	1:13,000 (1:10,000 to 1:16,000)‡	
25	Intramuscular	Rat	1:10,000	
20	Intravenous	Cat	1:64,000 (H)	1:16,000
20	Intravenous	Rabbit (3)*	1:64,000 (1:32,000 to 1:128,000) (H)‡	<1:32,000 (H)

*Number of animals used; others were single animals.

†Estimations were made with both *Staph. aureus* and *Str. hemolyticus*, except those marked (S) which were made with *Staph. aureus* only, and those marked (H) which were made with *Str. hemolyticus* which was more sensitive. The sign (<) means less than.

‡Range.

TABLE II. MINIMUM (AVERAGE) BACTERIOSTATIC CONCENTRATIONS OF CITRININ SODIUM

STAPH. AUREUS	STR. HEMOLYTICUS	AUTHOR AND REFERENCE
1: 34,000		Oxford ⁷
1:160,000		Timonin and Rouatt ⁸
1: 65,000		Timonin and Rouatt ⁸
1: 8,000		Robinson ⁹
1: 16,000		Chu
	1:18,000	Timonin and Rouatt ⁸
	1:40,000	Chu

same order as after intramuscular injection. Somewhat smaller doses (20 mg. per kilogram) than the smallest used in rats, given intravenously in a cat and three rabbits, gave much lower blood levels, the urine showing higher concentrations (Table I).

CIRCULATORY EFFECTS

When given intravenously in six dogs and two cats anesthetized with pentobarbital sodium, 30 mg. per kilogram, intraperitoneally, 7 mg. of citrinin per kilogram caused no appreciable changes in carotid blood pressure and heart rate. Doses of 10 mg. per kilogram produced a definite lowering of blood pressure with slowing of the heart, contraction of kidney, and increase in leg volume. Doses of 60 mg. per kilogram produced a marked fall (50 per cent) in blood pressure and slowing of the heart with dilatation of ventricles, followed by recovery in about twenty minutes. There was also contraction of a kidney with a simultaneous increase in leg volume. Doses of 100 mg. per kilogram produced

a similarly marked though prolonged fall in blood pressure, slowing of the heart, passive contraction of a kidney, and increase in leg volume with variable recovery. These changes suggested that the depressor action was due partly to peripheral vasodilatation and partly to direct injury of the heart. Very slow injections caused little or no changes in the blood pressure, heart and organ volume, even with the highest doses tried, that is, 100 mg. per kilogram.

Respiration was always increased in rate and amplitude during the fall of blood pressure. After atropinization, the blood pressure, instead of falling, was raised 20 or 30 mm. Hg with doses of citrinin 20 mg. per kilogram intravenously. Respiration was also increased during the rise of blood pressure. Thus, it would appear that the respiration was stimulated, in part at least, by the citrinin and not solely by the fall in blood pressure in unatropinized animals. The reversal of the depressor to a pressor effect confirmed a similar result reported by Ambrose.¹² The cause of this was not investigated, except that the same animals showed the same vasomotor reversal with mechohyl. If confirmed, it would suggest that citrinin possesses a nicotinic action like acetylcholine. A nicotine-like action would also explain the marked peripheral vasodilatation in rabbits' ears reported by Ambrose.¹² Further study of these effects of citrinin might be scientifically interesting, but was deemed unprofitable, since intravenous administration appeared chemotherapeutically unpromising.

MICROORGANISMS AND CATALYSTS IN VITRO

Bacteria.—Citrinin sodium inhibited *Staph. aureus* in dilutions of 1:16,000 in broth and *Str. hemolyticus* in dilutions of 1:40,000 in broth. Adding 50 per cent fresh human urine to the cultures augmented the bacteriostatic action to 1:40,000 for *Staph. aureus* and to 1:160,000 for *Str. hemolyticus*. Addition of serum had no effect on the bacteriostatic action. Generally, these minimum (average) bacteriostatic concentrations cannot be attained in the blood of living animals after introducing high doses into the nose and mouth irregularly from the alimentary canal (except in rats) but probably can be attained after intramuscular and intravenous injections. From one-half to one-third these doses might be prohibitive intravenously due to toxicity and these would probably not yield bacteriostatic concentrations. It is clear that citrinin is much weaker against these bacteria than penicillin. The results are summarized and compared with those of others in Table II. In general, my results indicate a lower order of bacteriostatic action than do those of Timonin and Rouatt⁸ and Oxford,⁷ although greater than Robinson's⁹ for *Staph. aureus* but higher than those of Timonin and Rouatt⁸ for *Str. hemolyticus*. These differences may be due to differences in sensitivity of the bacterial strains, although I tried several strains with about the same results.

Paramecium.—Solutions of citrinin sodium, 1:100, killed paramecia within five minutes; 1:500, in twenty minutes; 1:1,000, in thirty minutes; 1:2,000, in six hours; 1:5,000 did not kill even after twenty-four hours. Apparently this unicellular organism is rather resistant to citrinin.

Inorganic Catalysts.—Using Santesson's method,¹⁴ citrinin sodium concentrations of from 1:2,000,000 to 1:100 had no effect on the catalytic action of

platinum black (supernatant of 1 per cent suspension) for hydrogen peroxide. With collargol (0.1 per cent) and fuller's earth (1 per cent), stronger solutions of citrinin sodium, that is, from 1:100 to 1:500, aided the liberation of O_2 , if anything, while dilute solutions, below 1:500, produced no effects. Thus, the results with these inorganic systems do not indicate that citrinin is an important poison for the catalytic action tested. Using the same systems, Cutting, Halpern, and Armstrong¹⁴ found that penicillin in concentrations above 50 units per cubic centimeters always caused inhibition. This appeared to be due to impurities in penicillin, since the inhibition persisted after autoclaving.

PROTECTIVE ACTION AGAINST EXPERIMENTAL STAPHYLOCOCCUS INFECTION

Mice weighing from 18 to 20 grams were infected with twice the lethal dose of *Staph. aureus* by intraperitoneal injection of 0.5 c.c. of 1:5 dilutions of twenty-four-hour-old broth cultures. Two groups of fifty each were infected in that way. One group, used as controls, was treated by injecting 0.5 c.c. saline solution hypodermically at five-hour intervals day and night for three days from the beginning of infection. Another group was treated by the same procedure with 0.5 c.c. solution containing 0.5 mg. of citrinin sodium day and night for three days. All the animals died at about the same rate in both groups, leaving only two in each group on the third day. Thus, citrinin had no demonstrable protective effect in a fatal staphylococcal infection with high doses equal to about 25 mg. per kilogram of body weight or about one-half the toxic dose.¹⁰

Compared with penicillin, it seems obvious that citrinin is of no promise in systemic staphylococcal infection, but this might be different in local infections, as in abscess and furunculosis, where high concentrations could be applied directly.

SUMMARY AND CONCLUSIONS

1. Concentrations of citrinin sodium proposed in sprays, lotions, and ointments for local applications to mucous membranes and the skin were not demonstrably irritating or injurious and the slight to moderate irritation was not objectionable, as indicated by tests in animals and human individuals. High concentrations caused definite protoplasmic irritation or injury, but this was temporary and readily reversible.

2. Systemic absorption of citrinin was questionable or practically negligible for possible systemic or antiseptic actions of the drug in animals receiving high concentrations and doses by instillation into the nose or in the mouth.

3. Blood concentrations of from 1:4,000 to 1:32,000 (average, about 1:15,000) citrinin were demonstrable after introduction of high doses of citrinin into the ligated esophagus and stomach and all portions of the intestine of rats and also after intramuscular injection.

4. Generally, rapid intravenous injections of citrinin in doses of from 10 to 100 mg. per kilogram, caused immediate, although temporary, depressor effects, which increased with the dosage, due presumably partly to direct cardiac depression and partly to peripheral vasodilatation. The highest doses used

could produce dangerous sustained depression. Slow intravenous injections of citrinin produced no such depression. A reversal of the depressor to pressor action occurred in atropinized animals, similar to that of acetylcholine, in confirmation of others. While these phenomena may be worthy of further investigation, citrinin appears unpromising for intravenous use.

5. The minimum (average) bacteriostatic concentrations of citrinin in broth were 1:16,000 for *Staph. aureus* and 1:40,000 for *Str. hemolyticus*. The antibacterial action was unaffected by human serum, increased by fresh human urine, and decreased by heated urine. Paramecia were killed by concentrations of 1:2,000 and higher, but various inorganic catalysts were unaffected by concentrations of 1:100 and lower.

6. Experimental staphylococci infection in mice was not prevented with high doses (one-half the toxic) intraperitoneally. While citrinin is not comparable with penicillin in this systemic infection, this might be different in local infections where citrinin can be used in high concentrations. However, this remains to be determined.

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EFFECT OF SYNTHETIC VITAMIN K AND OF QUININE SULFATE ON THE PROTHROMBIN LEVEL*

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THE observation that the prothrombin level of the blood can be altered by various drugs is of clinical importance; however, it requires not only further study and confirmation, but also careful evaluation. In this paper, results are reported on the effect of synthetic vitamin K compounds and of quinine sulfate on the prothrombin of the blood, since it has recently been reported that the former¹ causes a hyperprothrombinemia while the latter² brings about a reduction of the prothrombin concentration.

THE INFLUENCE OF SYNTHETIC VITAMIN K ON PROTHROMBIN CONCENTRATION

The compounds studied were menadione (2-methyl-1,4-naphthoquinone), Synkayvite† (2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester tetrasodium salt), and Hykinone‡ (2-methyl-1,4-naphthohydroquinone sodium bisulfite complex). These were tested on rabbits, dogs, and man. A relatively large single dose was given and the prothrombin determined daily for several days. My quantitative method for the estimation of prothrombin³ was employed.

The results of giving large doses to rabbits and dogs are summarized by selecting typical experiments, which are presented in Table I. It appears obvious that there is no clearly demonstrable increase in prothrombin following large single doses of compounds having high vitamin K activity. This is contrary to the findings reported by Field and Link¹ and also by Shapiro and Richards,⁴ who noted a temporary decrease of the prothrombin time. Possible reasons for this difference will be discussed later.

A single dose of 40 mg. of hykinone given intravenously to a normal subject (27-year-old man; weight, 140 pounds) caused no alteration in the prothrombin level as seen by the results given in Table II.

Vitamin K in Idiopathic Hypoprothrombinemia.—Two years ago I discovered a consistent hypoprothrombinemia in a healthy robust medical student (22 years of age; weight, 188 pounds) who at no time has had any abnormal bleeding. The prothrombin level has remained practically fixed during the period in which he has been studied, as shown by the record given in Table III. It will be noted that 40 mg. of hykinone given intravenously did not alter the prothrombin.

The hypoprothrombinemia in this subject appears definitely to be con-

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TABLE I. EFFECT OF SYNTHETIC VITAMIN K IN LARGE SINGLE DOSES ON THE PROTHROMBIN LEVEL

PROTHROMBIN TIME IN SECONDS					
ANIMAL	WEIGHT (KG.)	DILUTION OF PLASMA			REMARKS
		UNDILUTED	1:5*	1:20†	
Rabbit 1	2.5	6	10	19	Control
2	2.5	6	10	19	120 mg. synkayvite orally
3	2.0	6	11	19	50 mg. hykinone intravenously
4	3.0	6	10	22	150 mg. menadione orally
Dog 1	6.5	6	10	20	50 mg. hykinone intravenously
2	8.0	6	11	21	Control
After twenty-four hours					
Rabbit 1		6	12	19½	
2		6	11½	19	
3		6	10	17	
4		6	10½	19	
Dog 1		6	11½	21	
2		6	11	19½	
After forty-eight hours					
Rabbit 1		6½	11½	18½	
2		6½	11	18½	
3		6½	9½	17	
4		6	10	17½	
Dog 1		6	10	22	
2		6	11½	20	

*Diluted with physiologic saline solution.

†Diluted with human plasma treated with aluminum hydroxide which removes prothrombin (actually component B³).

TABLE II

	PLASMA UNDILUTED	PLASMA DILUTED 1:5
Prothrombin time before hykinone (sec.)	12	27
Prothrombin time twenty-four hours after 40 mg. hykinone* (sec.)	12½	29
Prothrombin time forty-eight hours after 40 mg. hykinone* (sec.)	12½	27
Prothrombin time seventy-two hours after 40 mg. hykinone* (sec.)	12½	28

*Intravenously.

TABLE III

DATE	PROTHROMBIN TIME IN SECONDS	
	PLASMA UNDILUTED	PLASMA DILUTED 1:2
May 6, 1943	15½	19½
May 11, 1943	15½	21
May 13, 1943	15½	20
June 1, 1943	16	
Aug. 24, 1943	15½	
Nov. 19, 1943	15	19
Mar. 29, 1944	15½	20
June 7, 1944	18	
Aug. 11, 1944	17	
Dec. 15, 1944	15½	20
July 24, 1945	16	19½
July 25, 1945	16	20½
July 26, 1945	16	20

Gave blood for transfusion seventeen hours earlier

Gave blood for transfusion May 12 and 30

40 mg. hykinone intravenously

TABLE IV

	AGE (YR.)	DATE OF STUDY	PROTHROMBIN TIME IN SECONDS	
			PLASMA UNDILUTED	PLASMA DILUTED 1:2
Father	49	April 19, 1945	12	15
Mother	50	Dec. 15, 1945	16	22
Sister	16	Dec. 15, 1945	16	22
		June 25, 1945	17*	24
Brother F	21	May 28, 1943	12	15
Brother J	19	April 4, 1944	12	15
		May 17, 1945	12	15

*First day of menstruation.

genital, since it is present in other members of the family, as shown by the findings given in Table IV. From these results it can be concluded that the defect, or anomaly, is on the maternal side of the family and inherited both by the male and female. Data are lacking to show whether the male can transmit this characteristic fixation of the prothrombin level. Interestingly, the sister has had several episodes of severe uterine bleeding which apparently responded to vitamin K and small doses of snake venom. I did not have an opportunity to study the patient during the hemorrhagic attacks, since her home is on the other side of the state. Although she is taking vitamin K daily, the prothrombin has not been elevated to the normal level. It appears that the prothrombin concentration in three members of the family is fixed at a level a little lower than 50 per cent. A loss of blood seems to lower the prothrombin more easily than would occur in a subject with a normal prothrombin. Thus, giving two transfusions within eight days increased the student's prothrombin time to eighteen seconds which is equivalent to a prothrombin concentration of 35 per cent of normal. Likewise it appears that excessive menstruation caused the prothrombin to drop in the sister's blood and thus initiated a vicious circle. Vitamin K presumably can readily restore the prothrombin to the characteristic concentration but cannot elevate it beyond this fixed level.* It appears logical to designate this condition as congenital hypoprothrombinemia rather than idiopathic.

TABLE V. THE EFFECT OF QUININE SULFATE (0.33 GM. DAILY) ON PROTHROMBIN TIME

DAYS	PROTHROMBIN TIME IN SECONDS			
	SUBJECT 1 (AGE, 22 YEARS; WEIGHT, 160 POUNDS)	SUBJECT 2 (AGE, 21 YEARS; WEIGHT, 155 POUNDS)	SUBJECT 3 (AGE, 21 YEARS; WEIGHT, 150 POUNDS)	SUBJECT 4 (AGE, 22 YEARS; WEIGHT, 140 POUNDS)
0	12*	12½	11½	12
1	11½	12½	11½	11½
2	11½	12	11½	12
3	11	12½	12	12
5†	11½	12½	12	12
6	11½	12½	12	12
7	12	12½	12	12
8	12½	12½	11½	12

*The prothrombin time was also determined on plasma diluted with 4 volumes of saline solution. The results were entirely consistent with those of undiluted plasma.

†No determinations of prothrombin were made on the fourth day but quinine sulfate was taken.

*It has been found that the reduced prothrombin level is due to a lowered concentration of component B of the prothrombin complex. This same factor is diminished in dicumarol poisoning.

THE ACTION OF QUININE SULFATE ON PROTHROMBIN

The recent report of Pirk and Engelberg² that quinine in relatively small doses caused an increase in the prothrombin time seemed rather surprising, since hemorrhage is not a common complication even when this drug is given in large doses over long periods of time. A repetition of their experiments failed to show any reduction of prothrombin, as shown in Table V. The only difference in procedure was that Pirk and Engelberg employed Russell viper venom as the thromboplastin for the test. Whether this accounts for their findings is difficult to say, but it seems that it would have been judicious had they checked their results with my unmodified method for determining prothrombin since the latter procedure has been successfully used in all the known types of hypoprothrombinemia.

COMMENT

The terms hyperprothrombinemia and hypoprothrombinemia which are appearing with increased frequency in the medical literature require careful evaluation. Thus far hyperprothrombinemia has not been convincingly demonstrated either in man or in experimental animal. The results which have been interpreted as hyperprothrombinemia are based on the prothrombin time of highly diluted plasma. In such experiments, the disturbance of the physico-chemical equilibrium of the plasma by high dilution is not fully considered, and no attention is paid to the finding that prothrombin is not a single substance but a complex of two components and calcium as observed by me⁵ in 1943 and independently confirmed in part by Nolf⁶ in 1945.

The statement of Richards and Shapiro,⁷ "that only by the use of diluted plasma is it possible to detect the hyperprothrombinemia," cannot be accepted. I have demonstrated that the human being has only 20 per cent as much prothrombin as the dog and rabbit and therefore any significant increase of prothrombin in the human being could readily be detected by the prothrombin time of undiluted plasma. Actually it appears that the normal level is fixed and is not affected by even large doses of synthetic vitamin K.

Even if the prothrombin were increased above normal, it would not necessarily affect the coagulation time. Although the cow contains only 16 per cent as much prothrombin as the dog or rabbit, there is nothing to suggest that the cow is more subject to hemorrhage or that intravascular clotting is more prone to occur in dogs and rabbits. Both prothrombin and fibrinogen are normally present even in bovine blood in amounts far in excess of the requirement needed for normal coagulation; consequently further increases or decreases over a fairly wide range are not apt to affect coagulation or hemostasis significantly. The trigger substance in the clotting mechanism is thromboplastin.

Hypoprothrombinemia is clinically more significant, for if the prothrombin falls below 20 per cent, a patient is in danger of hemorrhage; however, as long as the vascular response remains normal and no trauma is sustained, abnormal bleeding is not likely to occur. I have seen a patient who, following a course of dicumarol therapy, had a prothrombin less than 10 per cent (from thirty-eight to forty-four seconds) for nearly two weeks without any evidence of bleeding. In

animals the prothrombin can be even more drastically reduced without actually causing hemorrhage, but such animals will bleed profusely when injured.

With the introduction of dicumarol into therapy and with the finding that other drugs can cause a decrease of the prothrombin of the blood, the necessity of a standardized test for determining prothrombin with the results expressed as per cent of normal has become urgent. The only test which has met these requirements is the one-stage method I have developed. In support of this is the recent paper of Hurn, Barker, and Magath.⁸

From the results obtained in this study, it appears unlikely that quinine in small doses given to normal individuals causes any demonstrable changes in the prothrombin of the blood. Whether the drug given in large amounts to patients with malaria will affect the prothrombin has not been determined. The suggestion of Pirk and Engelberg, that vitamin K be administered with quinine, had therefore better await more concrete evidence that such a practice is necessary.

While no specific studies on salicylates are reported in this paper, a comment on the hypoprothrombinemia due to this drug should be made. Sodium salicylate even when given at the level of 10 Gm. a day does not reduce the prothrombin to the hemorrhagic level in uncomplicated cases as Butt and associates⁹ have shown in a study of fifty-one cases. Some individuals nevertheless even on smaller doses of salicylates suffer a marked depletion of prothrombin. I have recently seen a child with a prothrombin time of forty-two seconds equivalent to a concentration of 8 per cent of normal following moderate therapeutic doses of sodium salicylate. The response to vitamin K intravenously (30 mg. hykinone) was rather slow. It seems fairly certain that in such patients some other dysfunction occurs whether it be of the kidney, liver, or some other organ.

The question naturally arises whether vitamin K should supplement the administration of salicylates. In the normal patient, vitamin K will restore and maintain the normal prothrombin level, but in these patients the level without vitamin K is not low enough to put them in jeopardy of hemorrhage. Whether small doses of menadione or its derivative will protect the small percentage of individuals who are supersensitive to salicylates has not been determined, but Richards and Shapiro⁷ have recently reported that in two patients of a series of twelve, synthetic vitamin K did not bring the prothrombin completely to normal. It seems, therefore, that probably the wiser course is not to depend on vitamin K, but rather to determine the prothrombin at frequent intervals on all patients receiving large doses of salicylates. If the prothrombin falls to a dangerous level, treatment with a water-soluble menadione derivative intravenously should be given immediately.

SUMMARY

1. Synthetic vitamin K compounds (menadione, synkayvite, and hykinone) given in relatively large single doses caused no demonstrable hyperprothrombinemia in rabbits, dogs, or man.
2. Vitamin K did not elevate the prothrombin of one individual who has a consistent level of approximately 45 per cent. Since the patient's mother and

sister likewise have the same concentration of prothrombin, it can be concluded that this trait is congenital and hereditary. The condition should therefore be called congenital hypoprothrombinemia.

3. Quinine sulfate in daily doses of 0.33 Gm. caused no decrease in the prothrombin of the blood.

4. The significance of hyper- and hypoprothrombinemia is discussed.

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DEPRESSION OF GAMMA GLOBULIN IN HYPOPROTEINEMIA DUE TO MALNUTRITION

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IN SEVERE hypoproteinemia due to uncomplicated malnutrition in man, the globulin fraction of the total plasma protein, as well as the albumin, may be depressed.^{1, 2} Although Weech and co-workers³ found normal plasma globulin levels in experimentally produced hypoproteinemia in animals, Madden and Whipple⁴ have emphasized that diet regulates production of both globulin and albumin. In the patient with nutritional edema described in this study there was a low plasma globulin which rose to normal as the hypoproteinemia was corrected by diet. The most striking changes were in the gamma fraction of the globulin. Since Cannoni and co-workers^{5, 6} have recently demonstrated that antibody response in rabbits may be influenced by experimentally produced hypoproteinemia, the immunologic implications of the present observations in a human patient are briefly discussed.

CASE REPORT

R. H. (History No. 119684), a 15-year-old white girl, was first examined on Nov. 11, 1944, and was carefully followed from April 1, to June 11, 1945.

History.—For one year before entering the hospital, the patient had noticed that she tired easily and that her ankles and legs were swollen at the end of the day. She came from a rural community and was a stepchild in a poverty-stricken family. Her diet had been markedly deficient, especially in proteins; beans and bacon constituted the only source of protein except for an occasional glass of milk which she received when she was able to attend school. She had had measles, mumps, chicken pox, and whooping cough. There was no history of nephritis. She had had no previous immunizations.

Physical Examination.—The patient was a thin white girl who did not appear acutely ill. There were no skin lesions or other obvious signs of vitamin deficiency. Slight tenderness could be elicited over the maxillary sinuses and a moderate amount of postnasal drip was seen on the back of the pharynx. There was slight pitting edema of both legs. The physical examination was otherwise normal.

Laboratory Findings.—Blood counts, repeated urinalyses, and stool examinations were normal. The plasma proteins were 3.1 Gm. per cent. Fractionation of the plasma showed 2.0 Gm. per cent albumin and 1.1 Gm. per cent globulin. Later protein studies including electrophoretic analyses are shown in Fig. 1, A and B. The blood N.P.N. ranged from 14 to 19 mg. per cent. The cephalin-cholesterol flocculation test was negative. A bromsulfalein test showed no dye retention after thirty minutes. Agglutination reactions with the typhoid-H antigen were negative in all dilutions.

Clinical Course.—When the diagnosis of hypoproteinemia was first made in November, 1944, the patient was placed on a high protein diet. Her home situation did not permit the carrying out of this regimen and she was brought back to the hospital in March, 1945, still edematous with plasma proteins of only 3.5 Gm. per cent. During the remainder of the study, she was on a high protein diet and the edema disappeared within a few weeks. By June 1, 1945, she had gained eight pounds and continued to improve.

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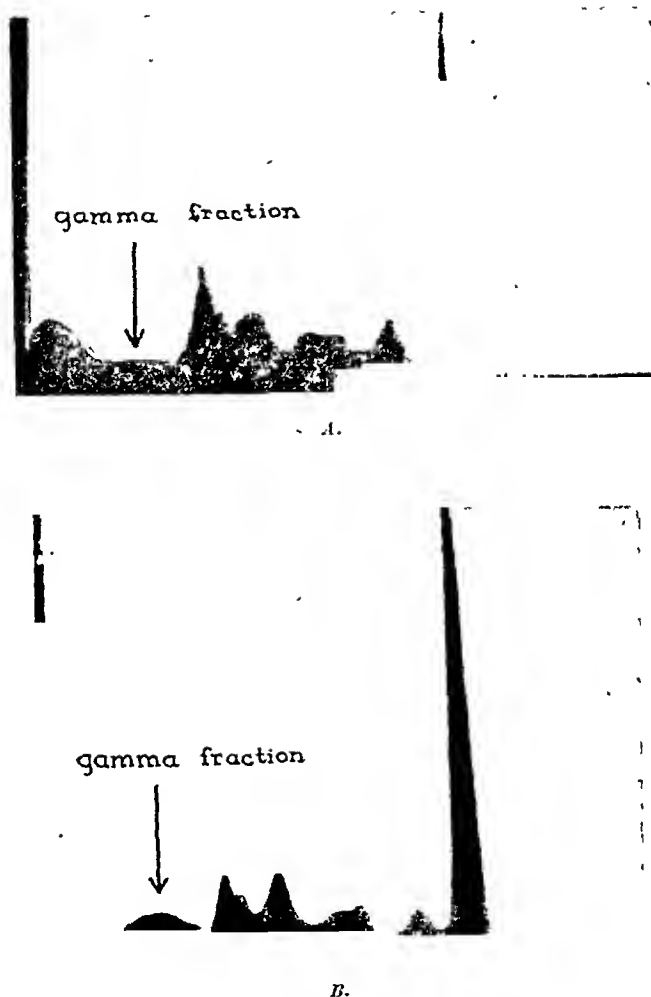


Fig 1—Electrophoretic patterns of the plasma of patient R. H. A, April 4, 1945, before high protein diet. B, June 11, 1945, after high protein diet.

METHODS

Dietary Regimen.—After preliminary investigation, the patient was placed on a diet furnishing approximately 100 Gm. protein, 125 Gm. fat, and 300 Gm. carbohydrate, providing approximately 2,700 calories. A typical day's menu of the recommended diet was as follows:

Breakfast

½ grapefruit
 1 serving Pettijohns
 2 eggs, scrambled
 2 slices whole wheat toast
 1 pat butter

Cream
 Sugar
 Jelly
 1 glass milk

Lunch

1 serving cream of pea soup
4 soda crackers
1 pat butter
1 peanut butter and jelly sandwich

1 serving Waldorf salad
2 sugar cookies
1 glass milk

Mid-Afternoon

1 serving baked custard

Dinner

1 large serving roast veal
1 serving mashed potatoes, gravy
1 serving Harvard beets
1 serving head lettuce salad,
salad dressing

1 slice whole wheat bread
1 pat butter
Jelly
1 serving vanilla ice cream
1 glass milk

This high protein, high caloric diet was maintained from April 4, 1945, through the entire period of observation. Exact calculations of the diet were not kept, because during most of this time the patient was not in the hospital; however, during the periods that she was at home, careful social service follow-ups insured the continuation of a high protein intake.

Immunizations.—Between April 21, 1945, and May 7, 1945, the patient was vaccinated against typhoid fever using 0.5 c.c. and two subsequent 1.0 c.c. injections of vaccine at weekly intervals. Bacterial vaccine* made from 1,000 million killed typhoid bacilli per cubic centimeter (Bio. 429 076384-A) was used. Agglutination reactions of the patient's serum with the typhoid-H antigen were carried out five weeks after the last injection.

Chemical Studies.—The total plasma proteins were determined by the Kjeldahl method. The fractional proteins were determined by the method of Campbell and Hanna,⁵ using sodium sulfite as the globulin precipitant. The electrophoretic measurements were made using a modification of the Tiselius apparatus described by Longworth.⁶ The cell with the tall center section was used and photographs were taken with the Philpot lens in the optical system. Oxalated plasma diluted with one part of buffer was dialyzed against 0.1 M lithium veronal buffer at pH 8.6. The measurements recorded in Table I were

TABLE I. CHEMICAL AND ELECTROPHORETIC MEASUREMENTS OF PLASMA PROTEINS

DATE	CHEMICAL ANALYSES (GM. PER CENT)			ELECTROPHORETIC ANALYSES (GRAMS PER CENT*)				
	TOTAL PROTEIN	ALBU- MIN	GLOB- LIN	α_1 GLOB- ULIN + ALBUMIN	α_2 GLOB- ULIN	β GLOB- ULIN	FIBRINO- GEN	γ GLOB- ULIN
11/18/44	3.1	2.0	1.1					
12/ 1/44	3.9	2.5	1.4					
3/23/45	3.8	1.8	2.0					
4/ 4/45	3.5	2.3	1.2	1.81 (51.8)	.49 (13.9)	.46 (13.1)	.55 (16.4)	.16 (4.7)
4/12/45	3.9	2.7	1.2	2.02 (51.9)	.59 (15.1)	.62 (16.0)	.52 (13.2)	.15 (3.8)
5/ 7/45	5.5	3.4	2.1					
6/11/45	5.1	—	—	2.54 (49.9)	.62 (12.1)	.66 (13.0)	.59 (11.7)	.65 (13.4)

*The relative percentages are in parentheses.

*Parke, Davis & Co., Detroit, Mich.

made from enlarged projections of the ascending limb with perpendicular lines marking off the separate fractions. Areas were measured with a planimeter.

RESULTS

A very low level of plasma gamma globulin was found before the patient's hypoproteinemia was corrected. Following a high protein intake, the gamma globulin increased from 0.15 Gm. per cent to 0.68 Gm. per cent (or almost the normal level). The changes in the albumin and all of the globulin fractions are shown in Table I. The changes in the electrophoretic patterns of the plasma are illustrated in Fig. 1, *A* and *B*.

The agglutination reaction of the patient's serum with the typhoid-H antigen was negative in all dilutions five weeks after the completion of the immunization.

DISCUSSION

This is the first reported case of uncomplicated nutritional hypoproteinemia with electrophoretic studies of the plasma proteins showing a diminished gamma globulin fraction which increased with a high protein intake. The author has studied one other patient with severe hypoproteinemia from chronic ulcerative colitis, and in this patient a relatively high gamma globulin was found which could probably be accounted for by the long-standing infection. Longworth and MacInnes¹¹ found very low gamma globulin concentrations in sera of patients with lipoid nephrosis where the diminution is probably accounted for, in part at least, by loss of protein in the urine.

The gamma fraction of the plasma globulin has been shown to contain many of the known antibodies; Kabat⁷ has reviewed the immunologic significance of this fraction of the plasma proteins. If the synthesis of gamma globulin is dependent on adequate protein intake, as this study suggests, antibody production may also be affected by diet. Cannon and associates⁸⁻¹⁰ have studied antibody production in rabbits made hypoproteinemic by a low protein diet and concludes that there is a lessened capacity of these animals to produce agglutinins as compared to rabbits on a well-balanced diet. They suggest that there may be a deficiency in the antibody containing fraction of the plasma globulin. In this connection it is of interest that a full course of immunization with typhoid antigen failed to cause an antibody response in the present patient.

SUMMARY

A case of hypoproteinemia due to malnutrition has been studied with electrophoretic determinations of the plasma proteins made both before and after a period of high protein intake. The plasma globulin as well as the albumin showed changes following dietotherapy, the most striking change being an absolute and relative increase in the gamma globulin fraction. The possible significance of this observation regarding antibody production and resistance to infection in malnutrition has been pointed out.

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THE INABILITY OF CYSTEINE TO INACTIVATE PENICILLIN IN THE PRESENCE OF BROTH AND BLOOD

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THE recent reports of Cavallito and Bailey¹ and Chow and McKee² on the inactivation of the antibiotic action of penicillin by cysteine aroused our interest in the possibility of incorporating cysteine in culture media for the inhibition of any penicillin present in body fluids to be cultured. It has been shown that penicillin in a body fluid will inhibit or prevent the growth of any penicillin-sensitive bacteria present in the specimen. Prompt inhibition of this bacteriostatic effect of penicillin in culture media is necessary to assure the growth of any penicillin-sensitive bacteria present.

Many investigators³⁻⁵ have shown that the addition of a sufficient amount of penicillinase prevents this action of penicillin immediately, thus allowing for unimpeded growth of any bacteria present. However, penicillinase is a bacterial product and has some disadvantages in its production, standardization, and sterilization. Cysteine hydrochloride is a chemical easily synthesized, weighed, and sterilized. This study is an attempt to evaluate the use of cysteine as an inactivator of penicillin in culturing body fluids.

On the basis of the work of Chow and McKee,² it was estimated that about 36 mg. of cysteine hydrochloride would inhibit the action of 30 units of penicillin in fifteen minutes. It was decided to use both penicillin X⁶ and G to determine whether there was any difference in the action of cysteine on these drugs. Accordingly, a series of six flasks was prepared. To each was added 5 c.c. of horse blood and 2.5 c.c. of penicillin solution containing 20 units per cubic centimeter. Three of the flasks contained penicillin X and the other three penicillin G. To one of each of the three flasks containing penicillin X and G was added 1 c.c. of a 6 per cent cysteine hydrochloride solution. One cubic centimeter of penicillinase† containing 10 units of penicillinase per cubic centimeter was added to two of the other flasks, one containing penicillin X and the other penicillin G. To all the flasks was added sufficient tryptose phosphate broth to make a total volume of 40 c.c. The final dilution of penicillin was 1.25 units per cubic centimeter in all flasks. Flasks 2 and 5 contained in addition 0.25 unit of penicillinase, and Flasks 3 and 6 contained 1.5 mg. of cysteine hydrochloride per cubic centimeter. Flask 1 with penicillin G and flask 4 with penicillin X served as controls. Samples of 0.4 c.c. were removed from each flask immediately on completion of the mixture for a control determination of the penicillin content.‡

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*The penicillin X was supplied by Lederle Laboratories, Inc., Pearl River, N. Y., as the calcium salt in ampules of 100,000 units containing about 75 per cent penicillin X.

†Penicillinase was supplied through the courtesy of Schenley Laboratories, Inc., Louisville, Ky.

‡Penicillin determinations were made according to a modification of the method of Randall,¹⁰ using an undiluted culture of *Bacillus subtilis*.

TABLE I. RESULTS OF ADDITION OF CYSTEINE TO PENICILLIN IN TRYPTOSE PHOSPHATE BLOOD BROTH

CONTENTS OF FLASKS					CONCENTRATIONS OF PENICILLIN (UNITS/C.C.) AFTER INCUBATION AT 37° C. FOR									
	VOLUME OF TRYPTOSE PHOSPHATE BLOOD* BROTH (C.C.)	PENICILLIN (UNITS)	PENICILLINASE (UNITS)	CYSTEINE (MG.)	CONTROL	15 MIN.	1/2 HR.	1 HR.	2 HR.	3 HR.	4 HR.	1-5 HR.	12-15 HR.	24 HR.
FLASK						0.025	0.025	0.025	0.025	0.025	0.025	0.312	0.312	0.312
1	37.5	50 (G)			1.25	0	0	0	0	0	0	0	0	0
2	36.5	50 (G)	10		0	0.025	0.312	0.156	0.156	0.156	0.156	0.078	0.078	0.078
3	36.5	50 (G)		40	1.25	1.25	1.25	1.25	1.25	0.025	0.025	0.025	0.312	0.312
4	37.5	50 (X)			1.25	0	0	0	0	0	0	0	0	0
5	36.5	50 (X)	10		0.312	0	0	0	0	0	0	0	0	0
6	36.5	50 (X)		40	1.25	0.025	0.025	0.312	0.312	0.156	0.156	0.156	0.156	0.156

*Five cubic centimeters of horse blood.

The flasks were then placed in an incubator at 37° C. for a total of twenty-four hours. At intervals of fifteen minutes, one-half, one, two, three, four, five, twenty-two and one-half, and twenty-four hours, 0.4 c.e. was withdrawn from each flask to ascertain the amount of penicillin present.

The results of this study are shown in Table I. It is apparent that the heat of the incubator destroyed some of the penicillin in the control flasks. Penicillinase produced prompt and complete inactivation of penicillin. On the other hand, cysteine inactivated only slightly more penicillin than was destroyed in the control flasks. Moreover, its action was delayed and incomplete when compared with that of penicillinase. There was no difference in the effect of cysteine on penicillin X or G.

Cavallito and Bailey¹ and Chow and McKee² have shown that certain amino acids antagonize the penicillin-inhibiting action of cysteine. The next problem was to determine whether the blood or broth, or both, used in our previous studies had any antagonizing effect on this action of cysteine.

To a group of four flasks was added 1 c.e. of 6 per cent cysteine hydrochloride solution. To Flasks 1 and 2 were added 2.5 c.e. of penicillin G containing 20 units per cubic centimeter. Flask 1 was made up to 40 c.e. with tryptose phosphate broth and Flask 2 with tryptose phosphate blood broth to the same volume. Fifty units of penicillin X plus 36.5 c.e. of physiologic salt solution were added to Flask 3. Flask 4 was similarly prepared with physiologic salt solution and blood.

From each flask 0.4 c.e. was withdrawn immediately for control determinations of the penicillin content of the flasks. The flasks were then incubated at 37° C. for a period of two hours and samples for penicillin assay were taken at fifteen and thirty minutes and at one and two hours.

The results are demonstrated in Table II. In the individual flasks containing blood and broth or physiologic salt solution and blood inactivation of penicillin was delayed and incomplete. In the flasks containing physiologic salt solution alone as a diluent, cysteine completely inhibited the action of penicillin after thirty minutes.

This study indicates that sufficient cysteine had been added to inactivate the quantity of penicillin employed in our work. The failure of cysteine to in-

TABLE II. RESULTS OF ADDITION OF CYSTEINE TO PENICILLIN IN TRYPTOSE PHOSPHATE BROTH AND IN PHYSIOLOGIC SALT SOLUTION WITH AND WITHOUT BLOOD

FLASK	CONTENTS OF FLASKS		PENICILLIN (UNITS)	CYSTEINE (MG.)	CON- TROL	CONCENTRATIONS OF PENICILLIN (UNITS/C.C.) AFTER INCU- BATION AT 37° C. FOR			
	VOLUME OF TRYPTOSE PHOSPHATE BROTH (C.C.)	VOLUME OF PHYSIOLOGIC SALT SOLUTION (C.C.)				15 MIN.	½ HR.	1 HR.	2 HR.
1	36.5		50 (G)	60	0.625	0.625	0.625	0.625	0.312
2	31.5 + 5 c.c. blood*		50 (G)	60	0.625	0.625	0.625	0.312	0.156
3		36.5	50 (X)	60	0.625	0.156	0	0	0
4		31.5 + 5 c.c. blood*	50 (X)	60	1.25	1.25	1.25	0.625	0.625

*Horse blood.

activate the penicillin in certain flasks in both experiments was apparently due to the antagonistic action of the broth and blood present. These fluids contain proteins and amino acid and their inhibiting action on cysteine in these studies substantiates the results of other investigators.^{1, 2}

Since cysteine did inactivate penicillin in physiologic salt solution, we were interested in determining whether the cysteine would have the same effect on penicillin in a synthetic medium* containing no proteins or amino acids. Coincidentally, we attempted to determine the effectiveness of smaller quantities of cysteine since Muir and Valley¹¹ and Hickey¹² have shown that 1 mg. of cysteine hydrochloride would inactivate about 400 units of penicillin in from thirty to sixty minutes using thioglycollate medium as a diluent. Chow and McKee² were able to inactivate 550 units of penicillin with 10 mg. of cysteine.

A series of nine flasks was prepared containing 2.5 c.c. of penicillin solution (20 units per cubic centimeter). To three flasks were added 0.12 mg. of cysteine hydrochloride dissolved in 1 c.c. of physiologic salt solution. Ten milligrams of cysteine hydrochloride were added to three flasks, and three flasks served as controls. Either tryptose phosphate broth or physiologic salt solution or synthetic broth was added to one flask of each of the groups. The total volume was 40 c.c. After removal of a sample for a control penicillin determination, the flasks were incubated at 37° C. for twenty-four hours. Samples for penicillin assay were withdrawn at fifteen minutes, one-half, one, two, and twenty-four hours.

The results are shown in Table III. We found that 0.12 mg. of cysteine did not inactivate 50 units of penicillin to any greater extent than did the heat of the incubator. Ten milligrams of cysteine did inactivate 50 units of penicillin in the physiologic salt solution within fifteen minutes. Compared to the controls, there was no greater inactivation of penicillin by cysteine, regardless of the quantity of cysteine, when the diluents were tryptose phosphate or synthetic nitrate broth.

TABLE III. RESULTS OF ADDITION OF CYSTEINE TO PENICILLIN IN TRYPTOSE PHOSPHATE BROTH, IN PHYSIOLOGIC SALT SOLUTION, AND IN SYNTHETIC NITRATE BROTH

FLASK	CONTENTS OF FLASKS					CONCENTRATIONS OF PENICILLIN (UNITS/C.C.) AFTER INCUBATION AT 37° C. FOR					
	VOLUME OF TRYPTOSE PHOSPHATE BROTH (C.C.)	VOLUME OF PHYSIOLOGIC SALT SOLUTION (C.C.)	VOLUME OF SYNTHETIC NITRATE BROTH (C.C.)	PENICILLIN X (UNITS)	CYS-TEINE (MG.)	CON-TROL	15 MIN.	1/2 HR.	1 HR.	2 HR.	24 HR.
1	37.5			50	0	1.25	1.25	1.25	0.625	0.625	0.156
2		37.5		50	0	1.25	1.25	1.25	0.625	0.625	0.156
3			37.5	50	0	1.25	1.25	1.25	0.625	0.625	0.156
4	36.5			50	0.12	1.25	1.25	1.25	1.25	0.625	0.156
5		36.5		50	0.12	1.25	1.25	0.625	0.625	0.312	0.156
6			36.5	50	0.12	1.25	1.25	0.625	0.625	0.312	0.156
7	36.5			50	10	1.25	1.25	0.625	0.625	0.312	0.156
8		36.5		50	10	1.25	0	0	0	0	0
9			36.5	50	10	1.25	0.625	0.312	0.312	0.312	0.156

*Synthetic nitrate broth:

K₂HPO₄ 0.5 Gm.
 CaCl₂ (anhydrous) 0.5 Gm.
 MgSO₄ 7H₂O 0.2 Gm.
 Glucose 10 Gm.
 KNO₃ 1 Gm.
 Distilled water to 1 liter

The failure of cysteine to inactivate penicillin in the tryptose phosphate broth corroborates our previous results. It has been shown that the action of cysteine is dependent on the hydrogen-ion concentration, with best results in pH 7 to 8. The failure of cysteine to inactivate penicillin in the synthetic medium was undoubtedly due to the fact that the mixture had a pH of 4. Bacterial growth was not sustained in synthetic nitrate broth when sodium bicarbonate was added to raise the pH. The pH of the physiologic salt solution was 6. This may be one of the reasons we were unable to corroborate the results of Muir and Valley¹¹ and Hickey¹² using comparable amounts of cysteine and penicillin. These investigators^{11, 12} were also able to inactivate penicillin with cysteine in thioglycollate broth. However, they allowed their cysteine-penicillin mixtures to stand for from thirty to sixty minutes before adding the mixture to the broth. In clinical work, this would not be possible.

Other investigators² have demonstrated that cysteine hydrochloride solutions are unstable at room temperatures, being oxidized to cystine. It has also been shown¹³ that different samples of one brand as well as different brands of cysteine hydrochloride vary in their ability to inactivate penicillin.

SUMMARY AND CONCLUSIONS

Ten milligrams of cysteine hydrochloride will inactivate 50 units of penicillin in physiologic salt solution. This inactivation is not observed in the presence of broth or blood or when the reaction of the medium is in the range of pH 4. Cysteine is not effective for the purpose of inactivating penicillin when it is used in culture media to which body fluids are added.

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LABORATORY METHODS

A PHOTOELECTRIC METHOD FOR THE DETERMINATION OF CHOLESTEROL IN URINE

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THE excretion of cholesterol in the urine has been studied in recent years by only a few investigators.¹⁻⁹ In our search for a suitable *clinical* method, preliminary studies showed that it is not possible to extract cholesterol directly from the urine. However, a complete hydrolysis is necessary in order to split the conjugations in which form at least part of the cholesterol is excreted in the urine. Further, in order to obtain correct colorimetric determinations, it is necessary to remove all foreign chromogens from the chloroform extracts. After these preliminary steps, the colorimetric determination of cholesterol in the urine follows the Law of Lambert and Beer and has great accuracy.*

METHOD

A complete twenty-four-hour urine specimen is collected and measured and its pH determined. An aliquot of 500 c.c. is used for one determination and is prepared in an extraction flask as follows:

1. Add from 60 to 65 c.c. concentrated HCl and mix.
2. Add 200 c.c. CHCl_3 (C.P.).

Connect the flask with the reflux condenser and bring the chloroform to a gentle boil for thirty minutes, thus carrying on hydrolysis and extraction at the same time. A continuous stream of small bubbles of chloroform should be in motion through the urine, but hard boiling of the chloroform must be avoided. The process needs constant watching and should be carried out at a temperature as close as possible to the boiling point of chloroform. Usually after twenty-five minutes of extraction, the completion of the hydrolysis is indicated by a marked change in color.

3. Cool to room temperature and transfer the contents of the extraction flask to a large separatory funnel. Rinse the extraction flask with a few cubic centimeters of CHCl_3 and add the washing to the separatory funnel.

4. Transfer CHCl_3 to a smaller separatory funnel.

5. Wash with three portions of 10 c.c. each of 10 per cent NaOH. If the last alkali washing is not practically colorless, continue up to six washings.

6. Wash three times with 10 c.c. portions each of distilled water. The alternate use of two separatory funnels facilitates steps 5 and 6. The separatory funnels should be rinsed with water between washings.

7. Collect in an Erlenmeyer flask, but carefully avoid carrying over any traces of water. The CHCl_3 extracts will have either a faint green or faint purple color.

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8. Stopper the flask and store in refrigerator for at least two hours or overnight.

9. Take 20 c.c. of the CHCl_3 extract (10 per cent of the original) and decolorize with 20 mg. decolorizing charcoal. Stir and filter through filter paper, Whatman No. 40, into a small beaker.

10. Heat on a hot plate, concentrate to approximately 4 c.c., and transfer to a small graduated cylinder. Wash the beaker with a few cubic centimeters of hot CHCl_3 twice more and add to the first 4 c.c. of concentrate. Bring the final solution to the 10 c.c. mark by the addition of a few drops of CHCl_3 .

11. Transfer to an Evelyn tube, stopper, and cool to 25°C . in a water bath. Add 4 c.c. acetic anhydride and 0.2 c.c. (4 standard drops) of concentrated H_2SO_4 . Do not remove from water bath while mixing. Place in diffuse light and take readings after exactly twenty minutes with filter No. 660 (center setting 61) of the Evelyn photoelectric colorimeter.

12. Calculate with K_2 factor 0.032 ± 0.002 .

EXPERIMENTAL

Investigation was made as to the influence on the Liebermann-Burchard reaction of substances present in the urine, either normally or under common clinical conditions, and as to the completeness of the extraction. The substances investigated were: (A) androgens, estrogens, creatinine, and bile acid, representing the normal group and (B) glucose and albumin representing the pathologic substances.

A. Androgens, Estrogens, Creatinine, and Bile Acid.—

1. Androsterone, trans-dehydro-androsterone, Δ^4 -androstenedione, testosterone, testosterone propionate, and estrone do not yield any color with the Liebermann-Burchard reaction. Consequently, an addition of these substances to the cholesterol-containing extracts did not influence the readings and did not interfere with the clinical applicability of the method.

2. Creatinine was not an interfering substance, even when an additional 600 mg. of creatinine per liter of urine were added to the normal urine. The readings obtained with filter No. 660 were not influenced in spite of an increase in the amount of unspecific brown color present.

3. Bile acids in the urine which may interfere with the Liebermann-Burchard reaction are excreted under normal conditions in only very small amounts; they were completely removed by the alkali washings in our procedure. We do not yet know whether this is enough to remove the increased amounts excreted by jaundiced patients.

B. Glucose and Albumin.—

1. The addition of as much as 10 Gm. dextrose per liter of urine did not influence the Liebermann-Burchard reaction. We therefore assume that the method can be applied to diabetic urine without advance preparation of the specimen.

2. When large amounts of albumin were present in the urine, as in nephrosis, an emulsion between the chloroform and albumin was occasionally formed

so that centrifugation became necessary to recover the chloroform. It is evident that working with an emulsified urine does not increase the yield. However, smaller amounts of albumin caused only a cloudiness of the urine during the process of extraction and do not interfere with the recovery of cholesterol. Therefore, the method is applicable in nearly all known clinical conditions.

C. The completeness of the chloroform extraction containing added known amounts of cholesterol was determined by re-extracting the discarded urine and the alkali washings. It was found that between 90 and 100 per cent of the amounts added to the original chloroform extract were recovered and only an unspecific brown chromogen remained in the otherwise discarded portions.

The addition to the urine of blood serum, the cholesterol content of which was determined separately by an adaptation of the Bloor method to the Evelyn colorimeter, gave yields which are shown in Table I.

TABLE I

NO.	CHOLESTEROL IN SPECIMEN (MG.)	CHOLESTEROL ADDED (MG.)	CHOLESTEROL RECOVERED (MG.)	RECOVERY (%)
1	2.8	2.3	5.3	108
	2.7	2.3	5.0	100*
2	1.3	1.3	2.3	77
3	1.6	2.8	4.4	100
	1.6	2.8	4.4	100*
4	0.55	1.0	1.55	100
5	4.0	3.3	7.9	118
6	0.4	0.1	0.5	100
	0.4	0.1	0.5	100*
7	2.2	2.3	4.6	104
	2.2	2.3	4.5	100*
8	1.1	2.0	3.1	100

*Specimens were divided into two equal parts and the determinations were made by two different workers.

DISCUSSION

The objections raised against the Liebermann-Burchard reaction are unwarranted as long as the necessary precautions and standard conditions indicated by several investigators^{2, 10-14} are observed. The Liebermann-Burchard reaction for blood cholesterol has been a valuable clinical test in our hands. Therefore, it appeared feasible to attempt the application of the Liebermann-Burchard reaction for the determination of cholesterol in urine.

We have shown that the determination of the chromogen in the urine is a quantitative indicator of its cholesterol content. Although the Liebermann-Burchard reaction is not an exclusive indicator for cholesterol, we found that 17-ketosteroids and other normal urinary contents do not give the Liebermann-Burchard reaction, that creatinine is destroyed, and that bile acids are removed. This leaves only the cholesterol as reagent in the chloroform extract, and the recovery experiments show that it actually is the sole source of the color reaction in our experiments.

It was found that the Schoenheimer and Sperry¹⁵ modification for precipitation of cholesterol esters in blood with digitonide was not necessary in urine, as

the addition of alcoholic digitonide did not increase the yield. This is probably due to the complete hydrolysis as the first step of our procedure.

This step is just the reverse procedure of that practiced by Bruger and Ehrlich⁹ who add as much as 10 per cent per volume of egg albumin to the urine to adsorb the cholesterol. Although these authors have been able to recover cholesterol from urine in this manner, the addition of egg albumin creates new factors of possible errors; these cannot be neglected in view of the small amounts of cholesterol in urine. We have shown that hydrolysis is an effective way to extract cholesterol completely and it requires fewer steps.

It may be argued that the amounts of cholesterol excreted in the urine under normal conditions are too small for exact colorimetric determination. However, the method described here is so sensitive as to reveal differences of 0.1 mg. cholesterol per specimen which is only a small fraction of the whole amount excreted. The amount of cholesterol excreted by normal individuals was found to be from 2.5 to 4.0 mg. per day.

SUMMARY

1. A simple technique is described for the photoelectric determination of cholesterol in urine.
2. The method is sensitive enough to detect variations of 0.1 mg. per sample of urine.

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ON THE ESTIMATION OF ARTERIAL CARBON DIOXIDE FROM SAMPLES OF CUTANEOUS (CAPILLARY) BLOOD

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ONE of the microgasometric methods for the estimation of blood gases developed by Scholander and Roughton² is designed for the determination of CO₂. This method, which departs from the principles employed in the analysis of O₂, CO, and N₂, depends upon the extraction of CO₂ from acidified blood by means of a partial vacuum. The use of a partial vacuum in the Scholander-Roughton syringe analyzer introduces certain technical difficulties which make the analysis of CO₂ less accurate than the analysis of the other blood gases. However, the accuracy obtained is sufficiently good to make the Scholander and Roughton method a valuable approach to a variety of physiologic problems where the quantity of blood for analysis may be limited.

The demonstration that cutaneous (capillary) blood obtained from the heated ear lobe was equivalent to arterial blood, with respect to the oxyhemoglobin saturation, suggested that, similarly, the arterial CO₂ content might be estimated from samples of cutaneous blood.¹ If true, this relationship would permit the estimation of arterial CO₂ contents without recourse to arterial puncture. In this report the accuracy of the microgasometric technique for CO₂ analysis is first defined and the feasibility of using cutaneous blood as a means for estimating the CO₂ content of arterial blood is then evaluated.

ACCURACY OF THE MICROGASOMETRIC TECHNIQUE FOR CO₂ ANALYSIS

The method employed in this study followed, in the main, that reported by Scholander and Roughton² in their original communication. The following minor modifications have proved useful in this laboratory:

1. The maintenance of vacuum in the syringe analyzer by means of a pure glycerin seal did not prove completely satisfactory. The sealing liquid was thickened by suspending 1 Gm. of gum tragacanth in 100 c.c. of glycerin (specific gravity, 1.249). This high viscosity suspension prevented all leaks.
2. Sealing of the analyzer cup by means of a latex-tipped toothpick proved satisfactory. The durability of the plug may be enhanced by using vulcanized latex. Maintaining the seal by manual pressure during the evacuation of the gas did not prove reliable. To avoid the necessity for manual pressure on the plug, the latex-tipped toothpick was mounted in a small wooden block to which was attached two elastic rubber bands. Another hinged wooden block was attached to the butt of the syringe barrel (Fig. 1, A). The toothpick was

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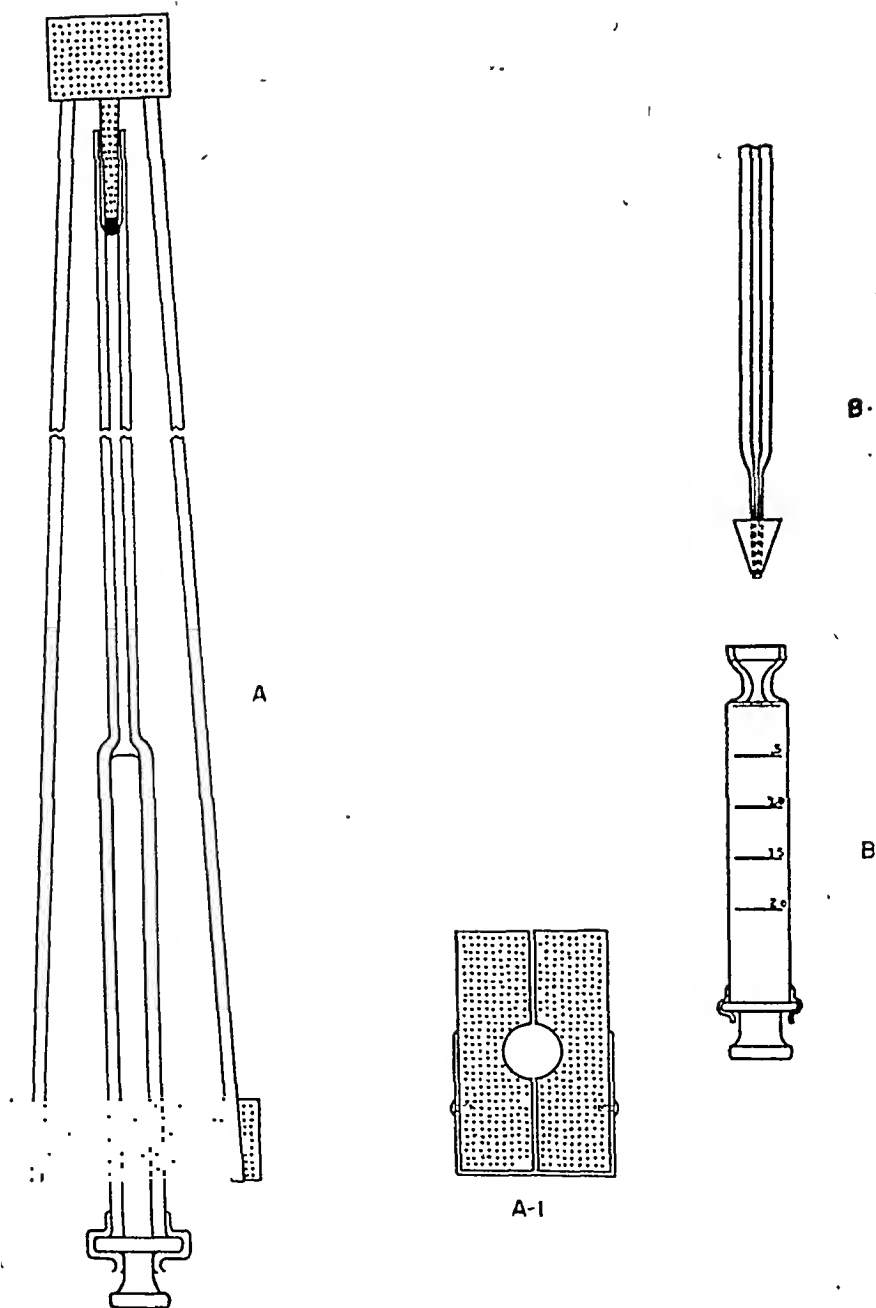


Fig. 1.

then seated in the cup and maintained firmly in position by looping the rubber bands over the retaining block (Fig. 1, A-1).

3. In accordance with the suggestion of Scholander and Roughton,² we have used a syringe analyzer with a longer capillary which permits the use of a 20 c.mm. sample (50 unit instead of the standard 30 units capillary). The increased volume of the sample of blood (0.02 instead of 0.012 c.c.) increased the accuracy of the method.

4. Foaming within the syringe analyzer often creates great difficulties which may be avoided largely by moistening the tip of the syringe plunger with a minute drop of caprylic alcohol when it is first prepared with the tragacanth-glycerol mixture. This small amount of caprylic alcohol is then deposited in a fine film over the wall of the syringe and reduces subsequent foaming. Persistent bubbles may be broken up readily by vigorous but guarded shaking of the analyzer in its long axis. It is important to note here that the microgasometric method for CO_2 is relatively much more difficult to master than the methods for O_2 , CO , and N_2 .

TABLE I. CO_2 CONTENT BY THE SCHOLANDER-ROUGHTON METHOD

DIFFERENCE BETWEEN PAIRED ANALYSES OF SIXTY-NINE SAMPLES OF BLOOD	
DIFFERENCE (VOL. %)	NUMBER OF SAMPLES
0.0	4
0.1	1
0.2	13
0.3	6
0.4	2
0.5	6
0.6	8
0.7	4
0.8	5
0.9	7
1.0	2 58 (84%)
1.2	1
1.3	3
1.5	2
1.6	1
2.0	2
2.3	1
2.4	1 11 (16%)
0.7 average difference	

The reproducibility of the analytic procedure may be evaluated from the results of a large number of duplicate analyses. In Table I is presented the difference between duplicate analyses of sixty-nine separate samples of blood. The average difference was 0.7 volume per cent; 84 per cent of the pairs differed by 1.0 volume per cent or less (total range, from 0.0 to 2.4 volumes per cent).

The accuracy of the Scholander-Roughton method may be evaluated by comparing results with those of the Van Slyke-Neill manometric method. In Table II are presented the results of duplicate analyses of eighteen separate blood samples both by the Van Slyke-Neill manometric and the Scholander-Roughton microgasometric methods. The Scholander-Roughton analyses aver-

TABLE II. COMPARATIVE VAN SLYKE-NEILL AND SCHOLANDER-ROUGHTON ANALYSES OF EIGHTEEN SAMPLES OF BLOOD

(1) VAN SLYKE- NEILL (VOL. %)	(2) SCHOLANDER- ROUGHTON (VOL. %)	(3) VAN SLYKE- NEILL - SCHOLANDER- ROUGHTON	(4) (2) \times 1.023	(5) (4) \cdot (1)
33.8	33.3	1.015	34.1	+0.3
47.0	45.0	1.045	46.2	-0.8
37.2	36.1	1.030	37.0	-0.2
42.1	41.8	1.005	42.8	+0.7
35.1	34.5	1.015	35.3	+0.2
43.1	42.4	1.015	43.4	+0.3
36.0	34.2	1.050	35.0	-1.0
59.3	56.3	1.050	57.7	-1.6
52.8	51.0	1.035	52.2	-0.6
44.5	43.2	1.030	44.2	-0.3
50.7	49.4	1.030	50.6	-0.1
47.0	45.2	1.040	46.3	-0.7
49.7	48.6	1.020	49.8	+0.1
50.2	49.7	1.010	51.0	+0.8
48.1	48.0	1.000	49.2	+1.1
46.6	45.5	1.025	46.6	0.0
48.4	48.6	0.995	49.8	+1.4
49.7	48.6	1.020	49.8	+0.1
Average		1.023	Average difference	0.6

aged 1.1 volume per cent less than the Van Slyke-Neill determinations. The average "i" factor (CO_2 by Van Slyke-Neill \div CO_2 by Scholander-Roughton) was 1.023 as compared with the "i" factor of 1.015 determined by Scholander and Roughton.² When corrected by use of the "i" factor, the average difference between results by the two methods was 0.6 volume per cent; 83 per cent of the bloods differed by 1.0 volumes per cent or less (total range, from 0.0 to 1.6 volume per cent).

EQUIVALENT OF ARTERIAL AND CUTANEOUS CO_2 CONTENTS

The comparison of the CO_2 contents of arterial blood with blood from the heated ear lobe was made in the same fashion as described in detail for the determination of oxyhemoglobin saturations.¹ An indwelling arterial needle permitted a sample of blood to be drawn from the brachial artery simultaneously with the sample from the heated ear lobe. The method originally described for oxyhemoglobin saturation employed a wet anticoagulant to obliterate the dead space in the collecting syringe. This liquid introduced an unknown dilution which did not alter the determined oxyhemoglobin saturations because they were expressed as percentages. However, this dilution would introduce an appreciable error into any determination of absolute content of CO_2 . In order to calculate the dilution factor, the collecting syringe was modified by fusing the collecting funnel directly to the syringe tip (Fig. 1, B). Each syringe was then calibrated by weighing it when dry and again when the syringe dead space and the funnel had been filled with the liquid anticoagulant (Heparin,* 1 mg. per cubic centimeter) up to a mark etched on the glass funnel. The volume of

*Lederle Laboratories, Inc., Pearl River, N. Y.

anticoagulant was then determined from the difference in dry and wet weights. The volume of the blood sample obtained was measured by the calibration of the syringe. The ratio of anticoagulant to blood permitted calculation of the dilution factor. With these syringes, the volume of anticoagulant varied from 0.083 to 0.12 c.c., and the samples of blood ranged in volume from 0.5 to 0.9 c.c., so that the dilution factors varied from 1.09 to 1.24.

The fused funnel-syringe requires a modification in the technique of transfer of the blood to the Scholander-Roughton pipette. This transfer may be effected anaerobically with ease by placing a small conical rubber tip over the end of the pipette (Fig. 1, B-1). This rubber tip may be inserted into the glass funnel to obtain a sample of blood. The tip is then removed and the sample transferred in the usual manner to the syringe analyzer.

TABLE III. COMPARISON OF CO₂ CONTENTS OF SAMPLES OF BLOOD OBTAINED SIMULTANEOUSLY FROM BRACHIAL ARTERY AND HEATED EAR LOBE (VOLUMES PER CENT)

(1) ARTERY VAN SLIKE- NEILL	(2) EAR LOBE SCHOLANDER- ROUGHTON × "i"	(3) (2)-(1)	(4) EAR LOBE CORRECTED SCHOLANDER- ROUGHTON × "i" 1.023	(5) (4)-(1)
47.6	46.9	-0.7	45.8	-1.8
44.4	45.9	+1.5	44.8	+0.4
50.2	52.4	+2.2	51.2	+1.0
48.1	49.2	+1.1	48.1	0.0
48.4	49.6	+1.2	48.5	+0.1
47.6	50.0	+2.4	48.8	+1.2
45.0	46.9	+1.9	45.8	+0.8
48.3	48.7	+0.4	47.6	-0.7
49.7	50.4	+0.7	49.2	-0.5
46.8	50.7	+3.9	49.5	+2.7
48.9	50.4	+1.5	49.2	+0.3
44.4*	45.1	+0.7	44.1	-0.3
Average		+1.4	Average difference	0.8

*Blood samples obtained at 30,000 and 35,000 feet pressure-altitude, respectively, in the decompression chamber.

The data obtained in twelve consecutive experiments are presented in Table III. The ear lobe samples were on the average 1.4 volumes per cent higher than the arterial samples. By coincidence, the factor by which the ear lobe values had to be multiplied to give the best possible approximation to the arterial values was almost equivalent to the reciprocal of the "i" factor (previously mentioned) derived. In other words, as shown in Table III, the two correction factors cancelled out. On this basis, the average difference between results by the two methods was 0.8 volume per cent (range, from -1.8 to +2.7 volumes per cent). In nine of the twelve comparisons, the difference was 1 volume per cent or less.

Since the oxygen saturation of blood from the heated ear lobe has been shown to be equivalent to that of arterial blood, the finding that under similar conditions the CO₂ content of ear lobe blood exceeds that of arterial blood requires explanation. Several possibilities which may be considered follow:

1. A constant error may enter in measuring the dilution due to anti-coagulant.

2. The ear lobe blood sample may be diluted by the introduction of tissue fluid containing larger amounts of CO_2 .

3. The erythrocyte volume of cutaneous blood under these experimental conditions may be somewhat less than that in arterial blood. If this state obtained, a "capillary" sample would contain a larger proportion of plasma and, therefore, a higher CO_2 content.

4. Although the oxygen saturation of blood from the heated ear lobe, as determined, indicated complete arterialization, this finding may be more apparent than real. For example, in the arterial range of oxyhemoglobin dissociation, no available analytic techniques will detect the minute change in saturation occasioned by a fall in oxygen tension of from 10 to 15 mm. Hg. However, a failure to attain complete arterialization would become evident with respect to CO_2 .

Whatever the explanation of these differences may be, the fact remains that the CO_2 content of blood from the heated ear lobe may be employed to estimate the CO_2 content of arterial blood.

SUMMARY

The Scholander-Roughton microgasometric method has been found to afford a usefully accurate means for estimating the CO_2 content of a minute sample of blood. This method applied to samples of blood from the heated ear lobe gives CO_2 values from which the CO_2 content of arterial blood may be estimated.

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BOOK REVIEWS

Bacillary Dysentery, Colitis and Enteritis. By *Joseph Felsen*, B.A., M.D., Director of Medical Research, Bronx Hospital, New York, N. Y.; Director of International and Pan-American Dysentery Registry. W. B. Saunders Company, Philadelphia. Price \$6.00. Cloth with 618 pages and 144 figures.

The author is to be congratulated for having accumulated between the covers of one book of 618 pages such a vast amount of information about every conceivable phase of this subject. It should be noted also that this is the first book to be published in the United States on this subject.

One is impressed by the broad coverage of the subject, by the unbiased presentation of data and opinions, by the author's exhaustive review of the literature, and by his unique adaptation of such a volume of information into its proper place in the presentation of the subject. One is impressed favorably by the author's vast personal experience and his active personal investigations in the subject. Such a background has enabled him to evaluate critically all information chosen for the book.

The historical aspects of bacillary dysentery are considered first. The disease has been traced from 480 B.C. to the present time, showing that it has always been a major disease of wars, a major military problem, and a constant and serious endemic problem in many parts of the world.

The type of information one would expect is found in the large section on epidemiology. However, in addition, one finds unusual information such as that found in sections on the survival of *B. dysenteriae* in soil and the gastric barrier in bacillary dysentery. There is much evidence indicating that the author has searched the literature exhaustively in reviewing the subject.

The clinical aspects of the disease are presented in considerable detail. The author has brought to bear on each subheading of this section the findings of many investigators interested in some certain phase of the disease. This is particularly true in the section on age incidence where the occurrence of this infection in the newborn period is stressed. Emphasis is placed particularly on the great variation in the symptomatology of the disease, from the asymptomatic or mild type to the rapidly fulminating and suddenly fatal type; also from the acute type with severe diarrhea to the mild type with constipation. Attention is also called to the atypical clinical forms of the disease. This is important because such unusual forms are not likely to be recognized by the clinician of average experience with this disease. It is important to know that in some cases the predominant symptoms suggest acute appendicitis, meningitis, pneumonia, or severe agranulocytosis.

The average observer of cases of this disease may recognize few complications. It is therefore timely and important that the author has presented the many complications which may occur. The relation of some of these to dysentery could easily be overlooked. Knowledge of the complications may also indicate in retrospect the true nature of a recent preceding infection.

The presentation of the bacteriology of dysentery is complete and up to date. The data in this section would complement greatly that found in our best textbooks on bacteriology at the present time. Much information has been accumulated in this section. Data are found regarding the various types of *B. dysenteriae*, the incidence of each in the etiology of dysentery, biochemical methods of classification of these microorganisms, their antigenic relationships, and the most recently suggested serological classification by Boyd.

The section on serological aspects of bacillary dysentery constitutes an important accumulation of information on this phase of the disease. The controversial views regarding the significance of serum agglutinins are presented in some detail. Data are presented showing that their presence is usually compatible with the disease, the convalescent state or the

carrier situation. Their absence is also compatible with a bacteriologically proved infection. Numerous data are presented showing a rise in agglutinin titers in the sera of human subjects as well as of rabbits following the administration of antigens of *B. dysenteriae*. Such data have been available previously only in the publications of various authors. These data have been brought together here and related to the patient with dysentery.

Various phases of prophylactic and curative therapy are considered. Much emphasis is placed on prophylaxis, as it should be. In a consideration of therapy the preponderance of data has to do with the use of various sulfonamides, as one would expect, since their curative effectiveness has been demonstrated repeatedly.

Chronic ulcerative colitis and chronic distal ileitis are presented as instances of chronic bacillary dysentery. The author has personally conducted follow-up studies of a number of these patients. He has demonstrated in 50 per cent of 124 such patients a positive culture, a previous history of the disease, the presence of bacteriophage for *B. dysenteriae*, or a combination of these findings. Other prevailing concepts of the etiology of chronic ulcerative colitis and chronic distal ileitis are also presented in all fairness. Differential diagnosis and treatment are given in considerable detail.

The appendix contains much information regarding technical methods used in the diagnosis of bacillary dysentery. This information includes a method for obtaining material for bacteriological culture from the intestine by sigmoidoscopic crypt aspiration, differential plating media, and cultural and serological identification of the types of *B. dysenteriae*. Similar information utilizing the most recent and best methods should appear in subsequent revisions of some of our standard textbooks on bacteriology.

The bibliography occupies ninety-five pages and is an excellent testament to the thoroughness with which the author has reviewed the literature dealing with every phase of the subject.

MERLIN L. COOPER, M.D.

Facial and Body Prosthesis. By *Carl Dame Clarke*, Ph.D., Associate Professor of Art as Applied to Medicine, School of Medicine, University of Maryland; Captain, Sanitary Corps, Army of the United States; Department of Moulage and Prosthetics, Army Medical Museum. The C. V. Mosby Company, St. Louis. Price \$5.00. Cloth with 200 pages.

This book is written by one who has had considerable experience in the field of prosthesis. The subject matter includes not only facial, but also body prostheses. These may be used for a short time until the defect can be corrected by surgery or, when surgical repair is not advisable, they may be used permanently. In the reproduction of a missing part of the body the basic technique is carefully considered and well illustrated. The advantages and disadvantages of the various materials, which have increased many-fold in recent years, are discussed. In the chapter on Repair of Cranial Defects by Cast Chip-Bone Grafts, the description of the technique could be condensed, and the surgical aspects are out of place in a book of this type.

When surgical repair of a body defect is not indicated, the prosthesis is an adequate substitute. The particular skills of the plastic surgeon, the dentist, and the artist are essential to the construction and wearing of a satisfactory appliance. Knowledge of the material contained in this book will help the novice avoid many disappointing results.

BERNARD G. SARNAT.

In the Doctor's Office. By *Esther Jane Parsons*, formerly Research Technician, Department of Biochemistry, College of Physicians and Surgeons, Columbia University; formerly Instructor in Medical Office Procedures, Paine Hall School for Medical Assistants, New York, N. Y. J. B. Lippincott Company, Philadelphia. Price \$2.00. Cloth with 295 pages.

RADIOACTIVE PHOSPHORUS AS A THERAPEUTIC AGENT. A
REVIEW OF THE LITERATURE AND ANALYSIS OF THE
RESULTS OF TREATMENT OF 155 PATIENTS WITH
VARIOUS BLOOD DYSCRASIAS, LYMPHOMAS,
AND OTHER MALIGNANT NEOPLASTIC
DISEASES

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THE ASSAY OF RADIOACTIVE PHOSPHORUS

RADIOACTIVE phosphorus has been used for the treatment of patients with various diseases since 1936. Lawrence and his associates¹ published in 1939 the first detailed report of therapeutic results obtained with radioactive phosphorus; the four patients discussed in this report all had leukemia. Since then, many observers²⁻¹⁷ have recorded additional experience in the treatment of leukemia and a wide variety of other disorders, principally malignant neoplastic diseases. These reports describe the effectiveness of this new therapeutic agent in a total of approximately 406 patients. Unfortunately, however, specific data in the form of case reports, charts, or graphs are given for only 140 of the subjects. It is the purpose of this communication to (1) review this literature critically, (2) summarize the experimental work which provided the rationale for the use of radioactive phosphorus, (3) describe our own experience accumulated during the past four years and based on the treatment of 155 patients who had various hematologic dyscrasias or malignant neoplastic diseases, and (4) evaluate the effectiveness of this therapy and compare it with older methods of treatment. We realize that four years of personal and nine years of over-all experience is insufficient for the final evaluation of radioactive phosphorus as a method of treating polycythemia vera and the chronic forms of leukemia. But even for these diseases it seems profitable to summarize existing information and draw tentative conclusions.

From the Edward Mallinckrodt Institute of Radiology and the Department of Internal Medicine, Washington University School of Medicine.

The authors wish to express their indebtedness to a large number of persons whose cooperation and help made these studies possible. Louis H. Hempelmann, M.D., initiated this survey but had to leave shortly thereafter for government service. Arthur Hughes, Ph.D., Harry Fulbright, Ph.D., Albert Schulke, and Harry Huth regularly supplied us with radioactive phosphorus often at considerable personal sacrifice of time and convenience. Reubenla Dubach, Ph.D., and Mary Lou Wheeler kindly made the measurements of radioactivity in tissues. Elizabeth Rice Sido was of invaluable aid in the hematologic studies. Finally, indebtedness is acknowledged to the many physicians who referred patients to us and made it possible for us to follow the patients at frequent intervals.

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For the sake of clarity, the various hematologic dyscrasias for which radioactive phosphorus has been used therapeutically will be treated separately. Under each such heading, the pertinent literature will be reviewed and our own data given. Material will be presented in the following order:

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I. MATERIAL AND METHODS

The diagnoses established for the 155 patients included in our own study were: polycythemia vera, 30; myelogenous leukemia, 39; lymphatic leukemia, 45; monocytic leukemia, 10; Hodgkin's disease, 6; lymphosarcoma (small cell type), 5; reticulum cell sarcoma, 3; giant follicular lymphoblastoma, 1; multiple myeloma, 8; Ewing's sarcoma, 3; malignant melanoma, 1; anaplastic carcinoma (primary site undetermined), 1; mycosis fungoides, 2; and xanthomatosis, 1. The diagnosis in every case was confirmed by sternal bone marrow aspiration or by lymph node or tissue biopsy. Of these 155 patients, 82 have died and 73 are still living. Post-mortem examinations were performed on 53 of the 82 patients who have died.

The radioactive phosphorus was prepared in the cyclotron of the Mallinckrodt Institute of Radiology by bombardment of red phosphorus with 12 million volt deuterons. The phosphorus was then synthesized into its dibasic sodium salt, according to the procedure used by Kamen,¹⁸ recorded in detail in a previous publication.¹⁹ Sufficient water was added to make an isotonic solution (15 mg. Na_2HPO_4 per cubic centimeter). The radioactivity of the phosphate solution was determined by means of a Lauritsen electroscope calibrated with a uranium standard.* The initial activity of a freshly prepared solution usually varied between 0.20 and 0.40 millieuries per cubic centimeter. The solution of radioactive phosphorus was used until its strength had decayed to from 0.04 to 0.05 millieuries per cubic centimeter.

The plan of therapy for all the diseases except polycythemia vera closely followed the "fractional method" outlined by Low-Beer, Lawrence, and Stone.¹² This consists of small frequent intravenous injections of radioactive phosphorus. In only a very few instances was the material given by mouth. The size of each dose given parenterally varied between 0.1 and 2.5 millieuries (100 and 2,500 microcuries). Patients were usually treated two or three times a week at first. As the white blood cell count either approached normal in the patients with leukemia or became subnormal in those in whom the white blood count was not elevated at the beginning of therapy, the dosage was decreased and the time interval between treatments was lengthened. Patients with polycythemia vera were given larger quantities of radioactive phosphorus at much less frequent intervals.

Therapy was governed principally by the changes produced in the peripheral blood. In patients with leukemia and polycythemia vera, an attempt was made to restore the cytology of the blood as nearly to normal as possible and to maintain this effect. Considerable individualization of treatment was necessary because of the great variation in response shown by different persons. The remissions induced in most patients were sufficiently good to permit interruption of therapy for a period of months. In a few instances it seemed advisable to continue regular injections of the radioactive phosphorus at intervals of several

*It has been discovered that calibration of radioactivity is made differently at the Massachusetts Institute of Technology than at the University of California and at Washington University. Furthermore, the values obtained at the first of these institutions differ from those made at the other two. A more complete discussion of these differences is given in the appendix to this paper, written by Dr. Martin D. Kamen. Whenever dosage is given in terms of millieuries, this discrepancy must constantly be borne in mind.

weeks. More active therapy was reinstituted whenever the changes in the peripheral blood indicated that the remission had ended, even though no symptoms developed at these times.

In the cases of lymphosarcoma, reticulum cell sarcoma, Hodgkin's disease, multiple myeloma, and the other malignant neoplastic diseases, in which the white blood cell count was not elevated, regulation of therapy was more difficult. Administration of radioactive phosphorus was continued in these instances until changes in the peripheral blood gave warning of a depression of bone marrow activity. In some patients, serial bone marrow aspirations were done to aid in regulating therapy. Patients with subleukemic leukemia were given amounts that were comparable to those required by leukemic subjects with elevated leucocyte counts.

On each visit to the clinic, symptoms were recorded, the patient was examined, and the following laboratory data were obtained: total red blood cell and white blood cell counts, hemoglobin, reticulocyte level, platelet count, and a leucocytic differential. While fixed cover slip preparations were made for permanent records, the differentials were made routinely with the supravital technique. The blood-counting equipment had all been standardized by the U. S. Bureau of Standards. Hemoglobin determinations were made by the Evelyn oxyhemoglobin method²⁰ on an Evelyn photometer which had been standardized by determinations of the oxygen combining power. Reticulocyte and platelet counts were done by the Dameshek method.²¹

II. RATIONALE FOR THE THERAPEUTIC USE OF RADIOACTIVE PHOSPHORUS

A. Definition of Radioactive Phosphorus and Description of Its Radiation Properties.—The nature of radioactive phosphorus, the means by which it is prepared in the cyclotron, the nuclear transformation which occurs when it disintegrates, and the characteristics of the radiation emitted have been described simply and clearly by Aebersold.²² In brief, to prepare radioactive phosphorus, ordinary stable red phosphorus is smeared on a copper target which is then placed directly in the path of a beam of high-speed deuterons (nuclei of heavy hydrogen) emitted by a cyclotron. As a result of this bombardment, a neutron enters the nucleus of a small fraction of the phosphorus atoms (Fig. 1). Stable phosphorus has an atomic mass of thirty-one—fifteen protons and sixteen neutrons, each of which has a mass of one. Since the mass of any atom is ordinarily written to the right and slightly above the atom's chemical symbol, stable phosphorus is referred to as P^{31} . When a neutron enters the phosphorus nucleus, the atomic mass is increased by one, and the resultant nucleus has a mass of 32. Radioactive phosphorus is, therefore, referred to as P^{32} ; its nucleus is unstable and the atom is radioactive. This new nucleus has the same number of protons (positive charges) as the nucleus of P^{31} ; thus, in the complete atom, there will be the same number of electrons (fifteen) revolving around the nucleus—one electron for each proton. The chemical identity of any atom is determined by the number of orbital electrons; P^{31} and P^{32} , therefore, are chemically identical. An atom of P^{32} eventually emits a beta-ray (an electron), whereupon one of the neutrons in the nucleus changes into a proton. The atomic mass of the nucleus

is not changed because protons and neutrons each have a mass of approximately one, but the positive charge on the nucleus is increased by one. This new nucleus is the nucleus of ordinary stable sulfur. The electron or beta-ray which is emitted when P^{32} is transformed into sulfur is the means by which radioactive phosphorus exerts its effect on tissues. These beta-rays have energies as high as 1.8 million electron volts; their average energy is approximately 0.6 million electron volts. The maximum range of penetration of these rays through the body tissues is approximately 0.7 centimeters. The half-life of P^{32} is 14.3 days, which means that within two weeks after administration any given dose of P^{32} will have lost half of its radioactivity by transformation into stable sulfur; within a month the activity will be one-fourth of the original value, etc. This rate of decay is short enough so that the radiation effects can be controlled, and yet it is sufficiently long so that a given tissue can be subjected to relatively prolonged, low-grade radiation. Thus, neoplastic or abnormally metabolizing cells can be subjected to a radiation effect throughout the whole of several mitotic cycles. If it is true, as has been suggested, that such cells are affected by radiation only during one phase of the mitotic cycle, then the prolonged, low-grade activity of P^{32} is an important theoretical advantage over x-ray therapy.

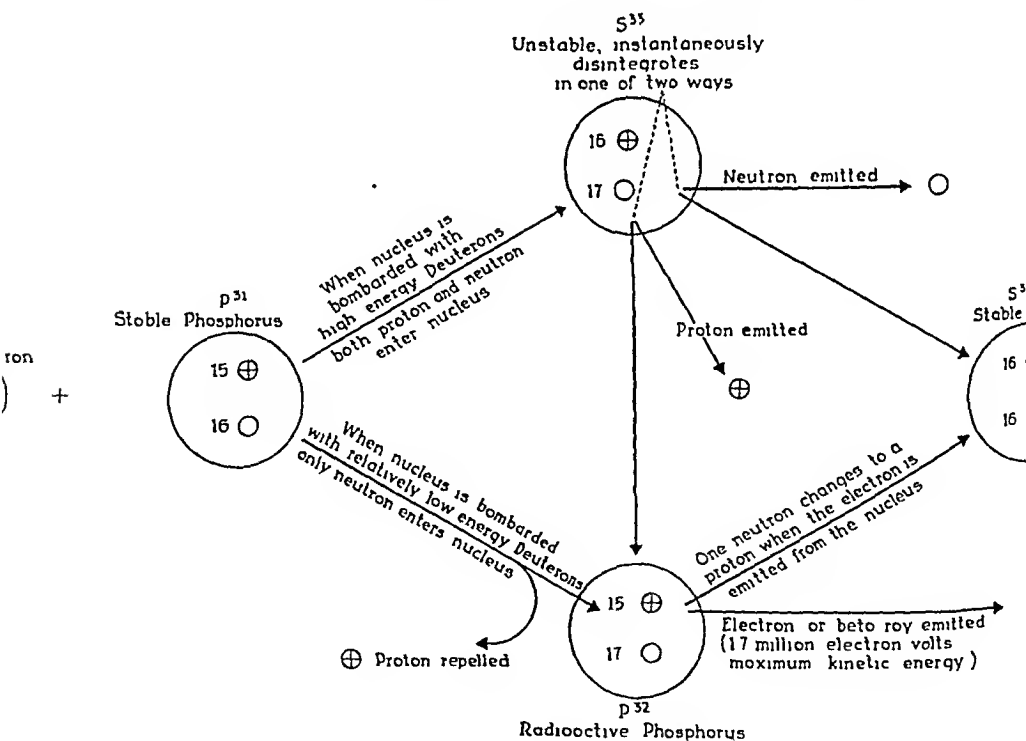


Fig. 1.—Radioactive phosphorus produced by deuteron bombardment of stable phosphorus (only nuclei are represented). Schematic presentation of nuclear changes involved in the production of radioactive phosphorus and its transformation into stable sulfur. Protons are marked with a plus because of their positive charge, while neutrons are left blank. The number of electrons which revolve about the nucleus (1) is equal to the number of protons in the nucleus and (2) determines the chemical identity of the atom. Since both stable and radioactive phosphorus have fifteen protons, they behave alike chemically.

When phosphorus is bombarded by deuterons in a cyclotron, only a small fraction of the atoms are made radioactive. The number depends on the number and energies of the striking deuterons. Actually, as the bombardment is carried out in the Washington University cyclotron, about one in every two million to five million atoms becomes P^{32} .

B. Definition of Millicurie and Relation to Roentgens of X-ray.—Since the dosage of radioactive phosphorus is always given in terms of millicuries, it is important that this unit of measurement be defined. A millicurie is that amount of any radioactive substance of which 37,000,000 atoms disintegrate per second¹² (see Dr. Kamen's discussion in the Appendix). Similarly, a microcurie is the amount which disintegrates at the rate of 37,000 atoms per second. This definition, it must be emphasized, does not take into account the type or energy of the radiation, both of which factors profoundly influence the physiologic effects of the radiation. For instance, radium emits alpha and gamma rays of high energy as well as beta-rays. One millicurie of radium, therefore, will produce different effects on tissue than will one millicurie of radioactive phosphorus. Consequently, one cannot assume that a millicurie of P^{32} will exert a radiation effect comparable to that of a millicurie of any other radioactive element or isotope.

An attempt has been made to translate these doses into terms with which clinicians and radiologists are more familiar. They have been related in particular to roentgens of x-rays.^{5, 9, 11, 22} For example:

1. If 1.1 microcuries of P^{32} remain in a kilogram of tissue until it has completely disintegrated, 1 roentgen equivalent of radiation will be delivered to that tissue.

2. If 1 microcurie of P^{32} remains in a gram of tissue for twenty-four hours, 43 roentgen equivalents of radiation will be expended in that tissue.

3. If 1 millicurie of P^{32} is retained for twenty-four hours by an adult weighing 70 kilograms, the effect will be equivalent to 0.6 roentgen equivalents of whole body radiation.

These relationships, however, are certainly not exact since x-rays are usually administered from an external source to the surface of the body and penetrate to a variable depth, while the beta-rays of P^{32} are emitted from phosphorus actually in the tissue.

C. Selective Uptake of Radioactive Phosphorus by Tissues and Cells.—Fundamental studies by a number of investigators²³⁻²⁹ have shown that P^{32} has a practical advantage over conventional methods of radiation therapy such as x-ray and radium in that when it is administered it is selectively withdrawn from the blood by certain tissues and cells. Fortunately, the tissues in which the greatest concentrations of radioactive phosphorus are deposited are usually those primarily involved in polycythemia vera, the leukemias, and the lymph node diseases. In general, this differential uptake of phosphorus by cells is dependent upon at least three factors: (1) the total amount of phosphorus in exchangeable form in the tissue, (2) the rate of turnover of phosphorus by the tissue, and (3) the rate at which new tissue is formed.

The extent to which the total exchangeable phosphorus in tissues influences the distribution of radioactive phosphorus is illustrated by bone. Bone has a

high inorganic phosphorus content and consequently takes up a large amount of the radioactive isotope. The effect of rate of turnover of phosphorus is illustrated by the marked difference in the accumulation of radioactive phosphorus in liver and brain. These two tissues have approximately the same phosphorus content, but liver takes up a large amount of phosphorus, whereas brain takes up relatively little due to the extremely slow rate of phosphorus metabolism by the brain. The effect of the rate at which cellular multiplication and new tissue formation occurs is illustrated by the fact that neoplastic tissue invariably takes up a great deal more phosphorus than does the same type of tissue in a normal state of growth. .

Lawrence and his associates^{26, 27} studied the distribution of radioactive phosphorus in the various tissues of normal and leukemic mice. A single dose of labeled phosphorus was administered to two groups of mice: one normal and one with lymphatic leukemia. The animals were sacrificed at various intervals, and the labeled (radioactive) phosphorus content of the tissues was determined. In Fig. 2 is shown the amount of radiophosphorus found in different organs expressed in per cent of the administered dose present per gram of wet tissue. There was only slightly more P^{32} in the bone and liver of the leukemic mice than in the corresponding organs of the normal animals. However, the lymph nodes and spleens of the leukemic animals took up from two to three times as much P^{32} as did these organs in the normal mice. This was interpreted as being due to the rapid laying down of new tissue as well as to the increased metabolic activity of the leukemic cells.

The distribution of radioactive phosphorus in the acid-soluble, phospholipid, and nucleoprotein fractions of tissues of normal and leukemic white mice has been studied by Tuttle and his associates.²⁷ A single dose of P^{32} was given intraperitoneally to each of a large group of normal and leukemic animals. The tissues were removed at varying intervals thereafter, the different organic phosphorus fractions were extracted, and the P^{32} content of each fraction was determined. In Fig. 3 is shown the distribution of the P^{32} in these different fractions. The nucleoprotein fraction of lymph nodes, spleen, and tumor tissue from the leukemic mice contained much more radiophosphorus than did the nucleoprotein fractions of corresponding organs in the normal animals. Thus, phosphorus (including radioactive phosphorus, if available) is taken up from the blood stream and used in the synthesis of nucleoproteins at a much more rapid rate by the leukemic cells than by cells in the corresponding normal tissues.

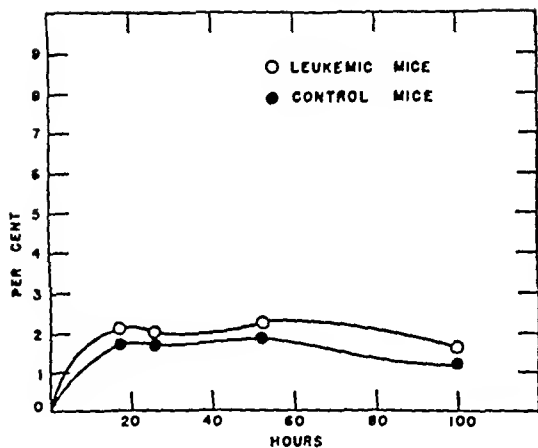
Marshak^{28, 29} devised a technique for the separation of cell nuclei from cytoplasm and then employed this method to study the relative uptake of inorganic radioactive phosphorus in the nuclei and in the cytoplasm of normal and malignant cells (lymphoma, sarcoma 180, and carcinoma 256). He found that the nuclei of the malignant cells accumulated more of the labeled phosphorus than did those of normal cells. Furthermore, the relative proportion of the administered phosphorus in the nuclei as compared to that in the cytoplasm was much greater in the malignant than in the normal cells.

The distribution of radiophosphorus in the blood of patients has also been followed. In leukemia, Tuttle, Scott, and Lawrence³⁰ observed a rapid uptake

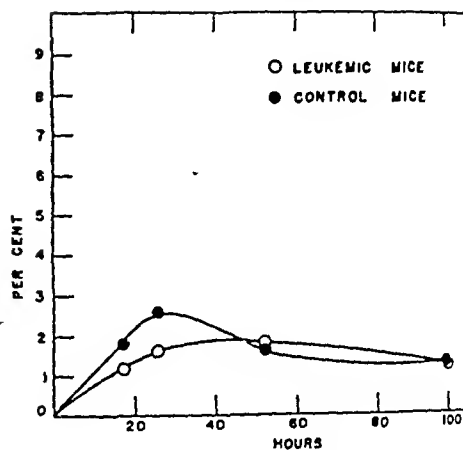
of the isotope by both red and white blood cells during the first twenty-four hours after administration. Within forty-eight hours, however, the concentration in erythrocytes began to decrease sharply, while that in the leucocytes continued to increase for an additional two to three days. At the end of nine days, the concentration per 100 cubic centimeters of white blood cells was between four and five times as great as in 100 cubic centimeters of erythrocytes. A determination made on the blood of one patient on the fifty-third day showed that this same ratio still held. It is of interest that phosphorus was present in the leucocytes during the first forty-eight hours largely in acid-soluble form. After that there was a gradual decrease in acid-soluble radioactive phosphorus together with a concomitant increase in the radioactivity of phospholipid and nucleoprotein fractions.³¹ The concentration of P^{32} reached in both white and red cells was higher following intravenous than following oral administration. Relatively greater concentrations of P^{32} occurred in the nuclei than in the cytoplasm of myeloid leukemic cells, but no differences were noted in the nuclei and cytoplasm of lymphoid leukemic cells. When large amounts of stable phosphorus (P^{31}) accompanied the administration, the concentration of P^{32} which occurred in cells was decreased. From a practical point of view, therefore, the P^{32} given therapeutically should be accompanied by the smallest possible amount of P^{31} . Marrow was found to retain radiophosphorus in higher concentrations than blood. These observations apply largely to leukemic cells. Retention of radioactive phosphorus by red blood cells of normal individuals and of patients with polycythemia vera may be greater than that of leucocytes during the first twenty-four to forty-eight hours; shortly thereafter the ratio becomes inverted because of a decrease in the red cell fraction.⁴ This decrease is apparently caused by a reduction in the radioactivity of the acid-soluble and nucleoprotein fractions; meanwhile, the P^{32} in the phospholipid fraction may actually increase slightly.

The distribution of radioactive phosphorus in the tissues of patients who had been treated with P^{32} has been studied by several groups of investigators. Leukemic patients who had received radiophosphorus shortly before death usually showed greatest concentrations of P^{32} in the bone marrow, lymph nodes, spleen, and liver.^{9, 32-35} These findings were similar to those previously described for leukemic mice. Those patients, however, who had been given radioactive phosphorus many days before death frequently had the greatest concentrations of P^{32} in bone. It appears, therefore, that the isotope is first utilized by the more rapidly metabolizing tissues or those most frequently infiltrated with leukemic cells and that later it finds its way to bone. In lymphosarcoma, a selective uptake of P^{32} by lymph nodes has usually been observed,^{8, 9, 12, 26} but in one patient studied by Erf and Lawrence³⁷ the mesenteric lymph nodes contained a smaller concentration than did many of the other tissues. In Hodgkin's disease, the findings have been variable and have apparently depended to some degree on the amount of fibrosis in lymph nodes. If the fibrotic change was advanced, small amounts of radioactivity were present in the nodes; if not, the lymph tissue contained concentrations comparable to those in rapidly metabolizing organs like liver and kidney.³⁷ Similar data have also been ac-

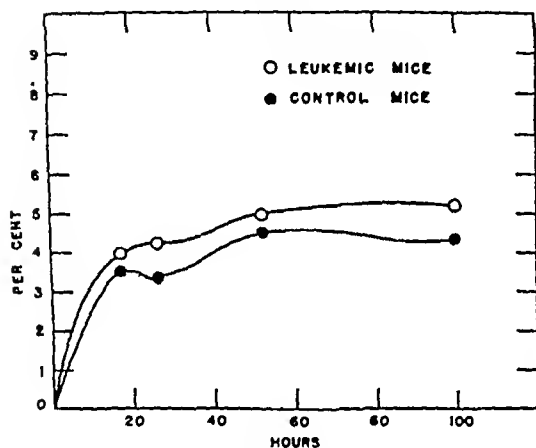
TOTAL ANIMAL



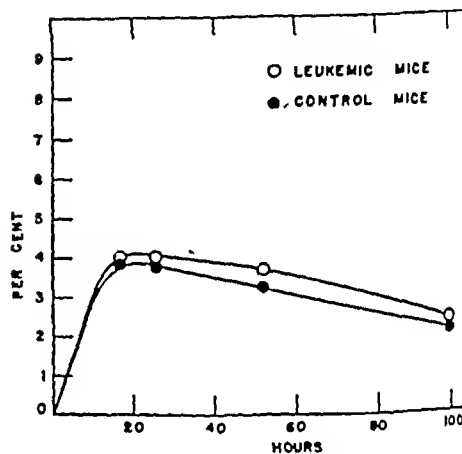
MUSCLE



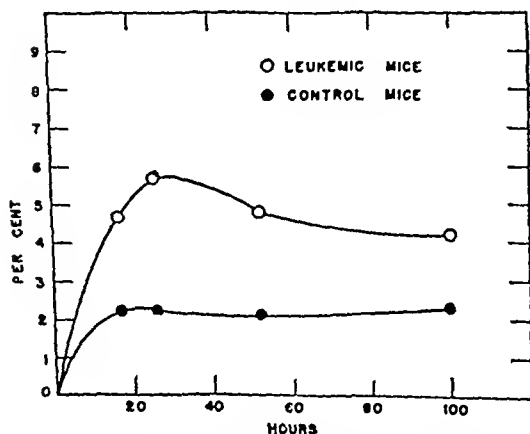
BONE



LIVER



LYMPH NODE



SPLEEN

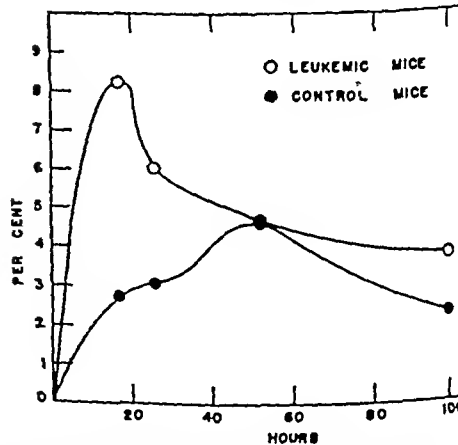


Fig. 2.—Distribution of radiophosphorus in the tissues of normal and leukemic mice. (Reprinted, with permission of Dr. John Lawrence, from J. Clin. Investigation 19: 267, 1940.)

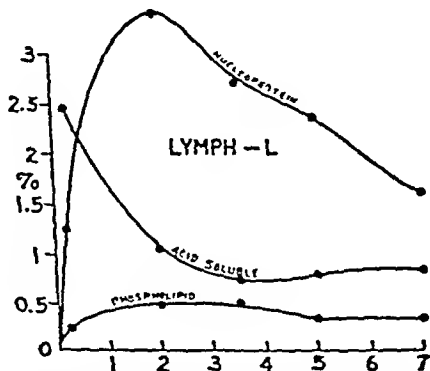
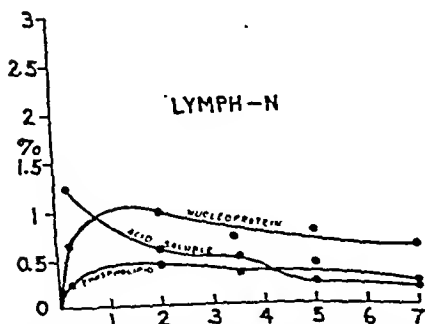
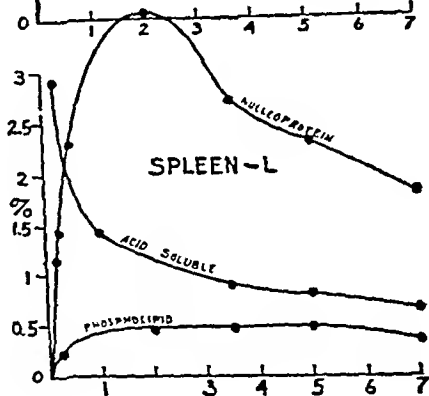
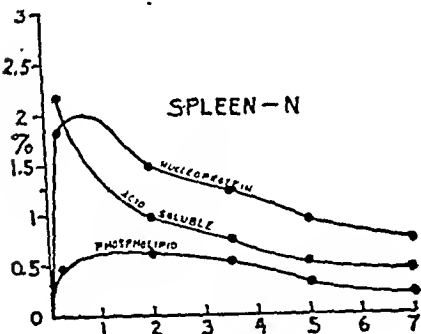
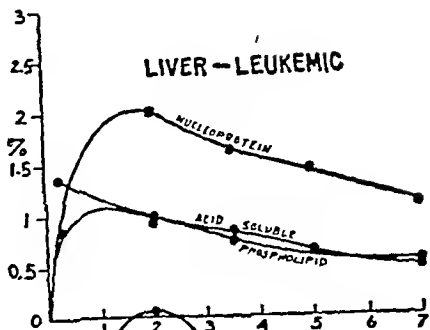
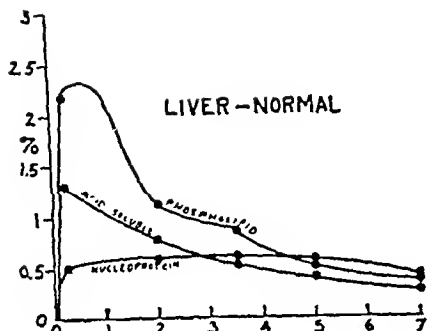
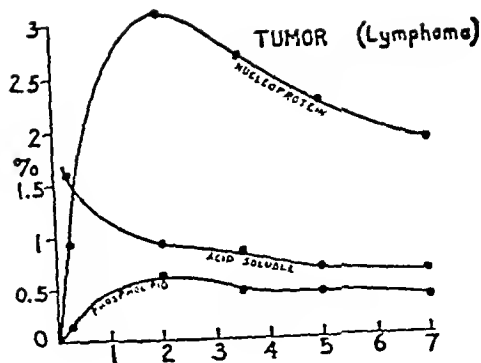


Fig. 3.—Phosphorus metabolism in neoplasms. The comparative uptake of labeled phosphorus in normal and leukemic mice. (Reprinted, with permission of authors, from J. Clin. Investigation 20: 57, 1941.)

TABLE 1. RADIOACTIVITY OF TISSUES OBTAINED AT AUTOPSY

PATIENT ^a	TOTAL DOSE (MC.)	TOTAL DURATION OF TREATMENT (WK.)	TIME SINCE LAST INJECTION OF P ³² (DAYS)	BONE MARROW	BONE	LIVER	SPLEEN
<i>Myelogenous leukemia</i>							
5	19.703	74	6	.064 (1)	-	.043 (4)	.032 (1)
10	69.863	26	60	.0232 (2)	-	.0218 (3)	.0148 (1)
13	44.957	52	1	.294 (1)	.161	.218 (3)	.232 (1)
14	44.236	58	7	.144 (1)	.067	.132 (2)	.128 (1)
19	18.317	22	12	.0473 (1)	.00825	.0470 (2)	.0279 (1)
20	4.532	1	3	.263 (1)	-	.088 (2)	.081 (1)
21	13.956	11	26	.0295 (1)	.0067	.0274 (2)	.0170 (1)
23	21.558	50	10	.0995 (1)	-	.0267 (2)	.0236 (1)
24	19.518	31	33	.00527 (1)	.00393	.00463 (2)	.00355 (1)
25	3.470	1½	1	.1180 (1)	.0523	.0857 (2)	.0650 (1)
<i>Lymphatic leukemia</i>							
3	31.440	147	14	.00905 (2)	-	.0103 (1)	.00624 (1)
17	8.446	5	5	.034 (5)	.049	.101 (1)	.071 (1)
19	23.649	31	21	.0437 (3)	-	.0535 (2)	lost
23	9.364	7	1	.0984 (4)	.0332	.173 (1)	.152 (1)
<i>Leucosarcoma</i>							
2	25.800	26	8	.0297 (4)	-	.0486 (2)	.0504 (1)
4	35.619	103	5	.0578 (1)	.0554	.0515 (3)	.0525 (1)
5	4.530	3	4	.0366 (3)	-	.0982 (1)	.0534 (1)
7	13.205	70	1	.145 (1)	.0025	.1097 (2)	.0664 (1)
9	17.256	29	8	.103 (4)	.165	.203 (1)	.180 (1)
11	10.088	4	2	.310 (3)	.374	.375 (1)	.350 (1)
12	6.858	4	3	.0504 (4)	.0126	.0827 (1)	.0636 (1)
15	9.560	6	7	.0520 (3)	-	.0570 (1)	.0443 (1)
<i>Monocytic leukemia</i>							
4	5.210	2	2	.120 (2)	.010	.124 (1)	.102 (1)
				.0915 (1)	-	.083 (3)	.086 (1)
6	4.964	1	4	.0620 (2)	-	.0535 (3)	.0640 (1)
7	4.504	½	1	.090 (2)	-	.104 (1)	.0727 (1)
<i>Reticulum cell sarcoma</i>							
2	15.081	18	10	.0190 (4)	-	.0501 (1)	.0422 (1)
<i>Hodgkin's disease</i>							
1	11.993	31	24	-	-	.0444	.008 (1)
<i>Multiple myeloma</i>							
3	8.660	7	1	.1688 (4)	-	.3442 (1)	.1949 (1)
4	5.002	2	4	-	-	.1020 (1)	.0660 (1)
5	10.908	16	61	.00268 (1)	-	.00109 (3)	.00119 (1)
<i>Melanosarcoma</i>							
1	7.748	4	5	.138 (1)	-	.0840 (3)	.0794 (1)

^aEach patient is identified by the same number in this table and in all tables summarizing data each disease.

cumulated for patients with neuroblastoma, seminoma, melanoma, Ewing's sarcoma, fibrosarcoma, osteogenic sarcoma, and carcinoma of the breast.^{9, 37} The radiophosphorus was preferentially retained by the tumor or tumor-infiltrated tissue in one case of seminoma, one of melanoma, and in five of osteogenic sarcoma; in one case each of Ewing's sarcoma and of neuroblastoma, it was retained as well by the tumor as by liver and kidney; the primary tumor in six patients with carcinoma of the breast concentrated the radioactivity less well than did either normal or metastatic lymph nodes in the same patient. While there are these and unquestionably other exceptions, leukemic and neoplastic tissues in general preferentially concentrate radioactive phosphorus. This tendency, however, is apparently not great enough to make radioactive phosphorus a thor-

EXPRESSED IN MICROCURIES PER GRAM OF WET TISSUE)

KIDNEY	MUSCLE	LYMPH NODE	BRAIN	LUNG	OTHER ORGANS
(3)	.023 (6)	.049 (2)			
(5)	.0079 (6)	.0238 (1)			
(4)	.098 (5)	-			.040 (Blood)
(3)	.032 (5)	-	.021		
(3)	.0216 (6)	.0278 (5)			.0059 (Blood)
(5)	.020 (6)	.063 (4)			
(6)	.0178 (4)	.0243 (3)			
(4)	.00995 (5)	-	.0025		
(4)	.00237 (5)	-	.0021	.0665	
(4)	.0308 (5)	-			
(4)	.00482 (6)	.00886 (3)	.00362		
(3)	.043 (4)	-		.055	
(4)	.0270 (4)	.0547 (1)			
(5)	.0447 (6)	.135 (3)	.0184		
(3)	.0249 (5)	-	.0120		.0207 (Intestine)
(4)	.0141 (6)	.0338 (5)			
(4)	.0237 (4)	.0193 (5)	.0120		
(3)	.039 (5)	-			
(3)	.091 (5)	-	.050		
(4)	.0856 (6)	.180 (5)			.0864 (Blood)
(2)	.0400 (6)	.0420 (5)			.01625 (Blood)
(5)	.0190 (6)	.0559 (2)		.01875	
(5)	.043 (6)	.116 (3)			.103 (Blood)
(4)	.0392 (6)	.0556 (5)			
(5)	.0263 (6)	.0474 (4)			
(3)	.0426 (5)	-			
(3)	.0077 (5)	-		.0211	.0603 (Tumor mass)
	-	.000006			
(3)	.0656 (5)	-	.0087		.1087 (Tumor)
(3)	-	-			.0310 (Intestine)
(4)	.000745 (5)	-			
(5)	.0194 (6)	.0966 (2)		.0615	.114 (Tumor)

oughly satisfactory therapeutic agent. Enough of the material is deposited in normal tissues and cells to limit the total dose which can be given without toxic manifestations. Even in leukemia, enough P^{32} cannot be given to destroy all leukemic cells without at the same time destroying erythroid and megakaryocytic elements.¹⁹

The distribution of radioactive phosphorus in various organs at the time of death was determined in thirty-two of the fifty-three patients in our series who were examined at autopsy. The results of these determinations are shown in Table 1. Approximately 1 gram samples of the fresh tissues removed at autopsy were weighed on an analytical balance. Each sample was then transferred to a Kjeldahl flask and digested with concentrated sulfuric acid and 30 per cent hydrogen peroxide. The clear digest was diluted with distilled water

to a volume of 50 cubic centimeters and various aliquots of this solution were measured into the glass well of a plunger or dipping type Geiger counter (Bale tube) until a dilution was found which gave approximately the same number of counts per minute as did a standard radioactive phosphorus solution of known activity. From this determination the radioactivity in microcuries per gram of wet tissue was calculated. Some of these patients had received frequent injections of P^{32} up to within a few days of the time of death, whereas some had not received any therapy for several weeks. If the interval between the last injection of radioactive phosphorus and death was greater than nine weeks, the tissues were not assayed for radioactivity.

In Table 1, the figure in parentheses to the right of the activity in microcuries represents the relative activity of various tissues. In determining these relative activities, only six tissues were considered: bone marrow, liver, spleen, kidney, muscle, and lymph node. The rating (1) indicates greatest activity, whereas the rating (6) indicates least activity.

In nine of the ten cases of myelogenous leukemia studied, the bone marrow had a greater activity than any other organ assayed, while in only one instance did a lymph node have the greatest activity. In contrast to this, in chronic lymphatic leukemia, the liver, spleen, and lymph nodes had greater activity than the bone marrow in most cases. In every case of chronic lymphatic leukemia the bone marrow had a lower activity than the liver. In the eight cases of leucosarcoma, the figures are more variable, but here again the liver and spleen tended to have somewhat greater activity than the bone marrow. Curiously enough, in every instance except one where a lymph node was assayed, the activity of the lymph node was less than that of bone marrow, liver, spleen or kidney. This was probably due to the fact that none of these patients at the time of death had significantly enlarged lymph nodes, and it was frequently difficult to find a gram of lymph node free from fat and connective tissue. Assays of the tissues from four patients with monocytic leukemia also gave variable results. The liver showed the greatest activity in two cases, the spleen in one, and the bone marrow in one. The one case of reticulum cell sarcoma studied showed more activity in the sarcomatous lymph nodes than in any other organ assayed. However, in the tissues of one patient with melanosarcoma, there was greater activity in the bone marrow than in the tumor.

The relative distribution of radioactive phosphorus among the different organs of the body depended, therefore, upon the type of neoplasm or leukemia and the degree of infiltration of any particular organ with abnormal cells. For example, at autopsy one patient with leucosarcoma showed considerable infiltration of the lungs with leukemic cells. On both gross and microscopic examination this infiltration was more marked at the left apex than at the left base. Assay of a portion of tissue from the left apex revealed an activity of 0.019 microcuries per gram of wet tissue, whereas tissue from the left base had an activity of only 0.015 microcuries per gram of wet tissue.

The distribution of radioactive phosphorus in the various tissues of patients with lymphatic leukemia indicates a somewhat less favorable differential uptake

of the radioactive material by the lymph nodes, spleen, and liver than was the case with the leukemic mice studied by Lawrence and his associates.²⁶ This may be due to the fact that our patients had received radioactive phosphorus for prolonged periods of time, whereas the mice were given a dose of P^{32} and the distribution in various tissues at 20, 40, 60, 80, and 100 hours was determined. The administration of P^{32} over longer periods of time may favor the accumulation of the material by those tissues which have a slower rate of phosphorus turnover.

D. Absorption and Excretion of Radioactive Phosphorus.—Absorption of P^{32} from the gastrointestinal tract of animals and of man has been studied by Chiewitz and Hevesy,^{38, 39} Hevesy and associates,⁴⁰ Lawrence and co-workers,¹ Erf and associates,^{5, 37, 41} and Cohn and Greenberg.⁴² From 15 to 50 per cent of orally administered radioactive phosphorus is excreted in the urine and feces both in normal individuals and in patients with leukemia during the first four to six days. Most of this amount is lost in the stool and represents unabsorbed rather than excreted material. Absorption, however, is good enough so that radioactive phosphorus can be given therapeutically by mouth; this route of administration has been used in several studies. It has become the practice of most workers who give P^{32} orally to assume that 75 per cent of any given dose is absorbed.

When radioactive phosphorus is injected intravenously into patients with leukemia, from 5 to 25 per cent of the isotope is excreted, mostly in the urine, during the first four to six days.³¹ Similar amounts are excreted by patients with polycythemia vera, but normal subjects regularly lose from 25 to 50 per cent within the same time period. After these first few days, the rate of excretion decreases in all subjects to less than 1 per cent of the injected dose per day. Erf and Lawrence suggest that the greater retention of intravenously administered P^{32} by patients with leukemia and polycythemia vera occurs because the isotope is more quickly fixed in the pathologic tissues and cells.

E. Summary.—Radioactive phosphorus emits beta-rays (electrons) which are capable of penetrating tissue to a depth of 0.7 cm. It differs from ordinary stable phosphorus only in that it is radioactive; its physiologic effects and its chemical properties are otherwise the same as those of the parent substance. Since this is so, however, relatively high concentrations of P^{32} in tissues and cells can be reached only if the amount of stable phosphorus given along with the isotope is kept at a minimum. The 14.3 day half-life of radioactive phosphorus permits steady irradiation of tissue for several weeks, yet is short enough so that radiation effects can be controlled.

Those tissues which have a high phosphorus content and which metabolize phosphorus rapidly selectively take up larger concentrations of radioactive phosphorus than do normal tissues. Since the radioactive isotope is built into nucleoprotein just as is ordinary P^{31} , those cells which are multiplying at the fastest rate use proportionately more of the P^{32} than do cells which are being produced more slowly. As a result, relatively high concentrations of radioactivity are reached in those organs principally involved in polycythemia vera, the leukemias, and the lymphomas. This preferential uptake, however, is not so great

that damage to normal tissues is avoided. In leukemia, for instance, one cannot give enough radioactive phosphorus to destroy all leukemia cells without also destroying erythroid and megakaryocyte elements.

When radioactive phosphorus is given by mouth, roughly 75 per cent is absorbed. After intravenous injection into patients, from 5 to 25 per cent is excreted within the first four to six days. The rate of excretion thereafter falls to less than 1 per cent per day.

III. POLYCYTHEMIA VERA

A. Review of the Literature.—There are seven papers^{3, 4, 10, 12, 14, 15, 16} in the literature which describe the clinical results obtained in the therapy of polycythemia vera with radioactive phosphorus. These papers are based on a study of forty-eight patients; for nineteen of these subjects specific clinical details are given either graphically or in case reports.

The first report was that of Lawrence³ in 1940, who recorded data on two patients. The first of these was a 42-year-old woman, previously treated with phenylhydrazine, who had an erythrocyte level of 8,900,000 cells per cubic millimeter on the day treatment with radioactive phosphorus was started. She was given orally 5.3 millicuries of P^{32} , and seventeen days later a second oral dose of 7.0 millicuries was given. The first significant drop in the erythrocyte level occurred exactly one month after the first treatment; three months after the first dose the erythrocyte count was down to 4,800,000 cells per cubic millimeter. The patient was greatly improved symptomatically. The second patient was a 59-year-old woman who had previously been treated by repeated phlebotomies; as a result, the erythrocyte count was only 5,900,000 cells per cubic millimeter at the time P^{32} therapy was started. The first dose consisted of 5.2 millicuries of radioactive phosphorus, and the second dose, given twenty-four days later, of 7.9 millicuries. Both doses were apparently given orally, although this was not definitely stated. Following this therapy, the erythrocyte count first rose, due, presumably, to the discontinuance of phlebotomies, and it was not until two months after the first treatment that the erythrocyte count dropped below the original level. Three months after the first treatment, the red blood cell count was down to 4,000,000 cells per cubic millimeter. These first two reported cases are summarized in some detail because the results obtained in them parallel closely those observed in all subsequent studies.

Fitz-Hugh and Hodes¹⁶ reported having treated eight patients with polycythemia vera, but no specific data regarding the dosage of radioactive phosphorus employed or the hematologic response obtained were given. The authors state that four of the eight patients were markedly improved both clinically and hematologically, one was unimproved, one was only slightly improved, and two had not been studied long enough to judge the effect. Two patients began to show hematologic relapse six and nine months, respectively, after the beginning of therapy. The authors further state that improvement may begin within two weeks of treatment, but full hematologic effect is rarely obtained until two to three months after therapy has been started.

Low-Beer, Lawrence,⁴ and Stone¹² mention that they have used P^{32} in the treatment of polycythemia vera for two and one-half years: fourteen patients were treated for a sufficient period of time to permit evaluation of the results. Data are given in this paper for only one patient, but the fourteen presumably include the two described in 1940 and the six for whom figures were recorded in a second report from the same laboratory.⁴ The method of treatment was to give an initial dose of from 5.0 to 7.0 millicuries and to repeat it three to four weeks later. In those patients with simultaneous involvement of the leucopoietic system, an initial dose of from 4.0 to 5.0 millicuries was followed by biweekly additional amounts to keep up the initial level over a period of from three to five weeks. The total amount administered to six of the patients during the first seven to fifteen months of treatment varied from 12.3 to 48.8 millicuries; most of this was given by mouth.⁴ The first signs of a satisfactory response were usually observed from six to eight weeks after treatment was started. Complete

or partial remissions were obtained in eleven of these fourteen patients, the remissions lasting from several months to two years.

Erf and Jones¹⁴ gave detailed data on eleven patients with polycythemia vera treated with radioactive phosphorus. The polycythemia was probably secondary rather than primary in one of these patients. The material was injected intravenously; dosages varied largely because the authors were unable to obtain the isotope at regular or specified intervals of time, and no specific planned regime was followed. From 1.55 to 11.5 millieuries of radiophosphorus were given during the initial period of therapy, which ranged from 11 to 117 days. Additional amounts were administered parenterally to two of the patients after intervals of from eight to fourteen months. Gratifying clinical improvement occurred in the ten subjects with true polycythemia vera, but one of these died suddenly following a large gastric hemorrhage twelve weeks after initiation of therapy. A transient anemia (3.4 to 4.0 million cells) developed in six of the patients. It is not possible to draw conclusions about the length of remission induced by therapy since only three subjects had been followed for more than one year at the time of publication and five had been observed for four months or less.

Warren¹⁵ treated three patients, two of whom were benefited. Data were recorded for only one patient, a 44-year-old woman with an initial erythrocyte count of 8,680,000 cells per cubic millimeter. She was given 3.0 millieuries of radioactive phosphorus intravenously, a second injection forty-six days later of 1.94 millieuries, and a third injection of 1.2 millieuries seventy-six days after the second injection. Seven months after the first treatment the red blood cell count was 4,520,000 and she was symptom-free. This complete remission lasted eleven months, after which interval she was given a fourth injection of 1.24 millieuries of P³². Three years after the first course of therapy she still felt well.

Hall, Watkins, Hargraves, and Giffin¹⁶ reported treating twelve patients with polycythemia vera. A satisfactory remission was observed in eight of these patients with complete relief of symptoms attributable to the polycythemia. The total dosage for a single course of therapy in these eight patients varied from 2.68 millieuries to 14.00 millieuries given in from one to three injections. The average dosage per course of treatment for these eight patients was 7.79 millieuries. The authors believe that the unsatisfactory therapeutic results obtained in the remaining four patients were due to inadequate dosage, even though from 6 to 14 millieuries were given in each instance. Even so, partial clinical improvement was noted in two of these individuals. Five of the twelve patients developed an anemia (3.4 to 4.0 million cells per cubic millimeter, from two to twenty-five months after the start of therapy; leucopenia (2,200 to 4,300 cells) also occurred in five subjects and thrombocytopenia (29,000 to 86,000 platelets per cubic millimeter) in four. Of particular interest is the fact that one patient, after a satisfactory remission of thirteen months' duration, died with the blood changes of acute leucopenic myelogenous leukemia.

From the foregoing summary of the literature, it is apparent that there is substantial agreement that excellent clinical and hematologic remissions can be obtained in the majority of patients having polycythemia vera by therapy with radioactive phosphorus. Very few of the reported cases, however, had been followed for a sufficiently long period of time to permit evaluation of the duration of remissions induced in this manner. Another feature which requires clarification is the proper dosage and method of administration, concerning which there is considerable diversity of opinion in published reports.

B. Analysis of Results Obtained in Treating Thirty Patients With Polycythemia Vera.—During the last four years, thirty patients with polycythemia vera have been treated with radioactive phosphorus in the Mallinckrodt Institute of Radiology. In addition to the routine blood and bone marrow studies, the following laboratory data were obtained on these patients: (1) x-ray of chest, (2) electrocardiogram, (3) circulation time, (4) basal metabolic rate, and (5) blood volume using Evans blue dye according to the method of Gibson and Evans,¹⁷ as modified for use with the photoelectric colorimeter.¹⁸ In those

TABLE 2. HEMATOLOGIC DATA ON THIRTY PATIENTS WITH

PATIENT	AGE AND SEX	TOTAL RADIOACTIVE PHOSPHORUS FOR EACH COURSE OF TREATMENT (MC.)	DURATION OF COURSE OF THERAPY (DAYS)	TIME FROM FIRST TREATMENT TO INITIAL DROP IN R.B.C. LEVEL (DAYS)	R.B.C. COUNT AT TIME THERAPY WAS BEGUN (MILLIONS PER C.M.M.)
1 ^a	37, F	7.03	34	40	8.59
2	53, F	5.24	69	40	7.33
		16.88	429	34	6.66
3	45, M	7.41	112	30	6.34 ^a
		1.80	1	27	6.35
		2.56	1		6.45
4	60, F	5.74 ^b	2	30	7.83
5	42, M	5.74	66	88	9.00
6	38, M	8.44	70	41	6.65
7	56, F	13.25	75	33	7.00 ^a
8	65, F	5.66	29	40	8.11
9	44, M	6.71	92	60	7.91
10	38, F	10.50	49	61	7.15 ^a
11	50, F	4.90	43	28	7.91 ^a
		2.53	32	28	5.75
12	23, F	7.78	139	30	7.56
13	47, F	6.92	120	47	6.47 ^a
14	57, M	5.01	87	60	8.01
		3.62	56	112	6.80
15	66, F	5.17	2	48	7.57
16	59, M	4.07	1	58	7.98
17	56, M	4.03	1	41	6.02 ^a
		3.00	21	30	6.38
18	63, M	4.14	44	83	5.78 ^a
		3.58	40	92	5.87
19	56, F	7.72	286	--	5.88 ^a
20	28, M	3.64	1	29	8.50 ^a
21	65, M	9.36	338	-- ^c	5.75 ^a
22	59, M	3.78	44	44	7.65
23	49, M	4.79	65	-- ^c	6.16 ^a
24	61, M	3.96	1	--	8.18 ^a
25	66, M	3.54	1	42	7.08
26	51, F	4.69	32	44	9.04
27	32, M	4.03	1	27	6.59 ^a
28	63, M	3.50	1	-- ^c	8.15 ^a
29	33, M	3.44	1	21	7.09 ^a
30	39, M	3.63	1	--	5.33 ^a

^aPatient treated by phlebotomies or phenylhydrazine up to the time P³² therapy was begun. It is probable that there was greater erythroid activity than was suggested by the red blood cell count.

^b7.66 millicuries given orally which is equivalent to 5.74 millicuries intravenously (assuming 75 per cent absorption from the gastrointestinal tract).

cases in which, after evaluation of all the above tests, there was still some doubt as to whether the patient had polycythemia vera or secondary polycythemia, arterial oxygen saturation was determined by the method of Van Slyke and Neill,⁴⁵ as described in Peter and Van Slyke's *Quantitative Clinical Chemistry Methods*.⁴⁶ It is felt that the diagnosis of polycythemia vera was conclusively established in every patient included in this series.

1. *Effect of Therapy on the Cellular Elements of the Blood:* Hematologic data on these patients are summarized in Table 2. A study of the figures in column 3 reveals that our patients were given smaller doses of P³² than have

CYTHEMIA VERA TREATED WITH RADIOACTIVE PHOSPHORUS

TEST R.B.C. COUNT LLIONS PER C.MM.)	PLATELET COUNT AT TIME THERAPY WAS BEGUN (PER C.MM.)	LOWEST PLATELET COUNT AFTER THERAPY (PER C.MM.)	W.B.C. COUNT AT TIME THER- APY WAS BEGUN (PER C.MM.)	LOWEST W.B.C. COUNT AFTER THERAPY (PER C.MM.)	DURATION OF HEMATOLOGIC REMISSION (FROM TIME R.B.C. COUNT FELL BELOW 5.5 M. UNTIL IT AGAIN ROSE ABOVE 6.0 M.) OR INTERVAL BETWEEN COURSES OF TREATMENT (MO.)
3.29	2,490,000	358,000	15,450	3,400	26+
4.77	264,000	230,000	18,750	4,200	3 ^d
4.81	839,000	517,000	6,750	17,300	
4.59	3,685,000	258,000	21,600	3,950	10
5.16	940,000	345,000	13,350	6,600	9
5.59	1,490,000	640,000	14,850	7,200	2+
3.35	2,036,000	29,000	43,700	1,100	30+
3.19	1,010,000	191,000	5,400	2,500	21+
4.75	458,000	454,000	6,400	3,200	3+
					(No further follow-up)
3.37	2,080,000	287,000	13,500	1,500	33+
2.97	1,233,000	107,000	11,100	1,200	25
4.77	1,266,000	626,000	15,750	7,050	6+
3.27	2,016,000	250,000	5,000	1,650	32+
4.34	1,818,000	164,000	11,600	4,100	19
4.30	1,250,000	469,000	10,300	3,650	2+
4.99	2,600,000	744,000	12,550	5,450	11
5.60	3,300,000	476,000	17,200	7,150	13+
5.12	820,000	364,000	9,900	4,300	5
4.46	490,000	392,000	7,700	3,800	9+
3.50	1,582,000	153,000	9,650	950	15+
4.47	1,607,000	191,000	10,400	1,550	15+
4.46	1,250,000	260,000	12,000	1,150	8
3.84	376,000	182,000	5,350	3,700	9+
3.54	1,543,000	724,000	13,300	5,250	10
5.09	740,000	--	11,850	7,200	3+
5.55	2,610,000	964,000	6,700	5,200	2+
3.70	1,802,000	343,000	12,250	7,600	12+
5.18	1,502,000	884,000	11,250	6,700	1+
3.95	--	274,000	7,100	3,950	7+
4.92	1,438,000	357,000	16,200	7,600	5+
5.15	2,160,000	1,590,000	14,200	7,800	1+
4.21	553,000	106,000	10,550	4,700	5
4.45	1,503,000	241,000	10,650	1,970	2+
4.65	1,931,000	715,000	9,250	6,300	2+
5.09	689,000	395,000	9,450	3,950	1+
6.60	790,000	482,000	15,650	7,750	1+
4.72	5,080,000	--	20,550	8,400	-

*Phlebotomies were done in addition to giving patient P²² therapy. No more than four phlebotomies were done in any patient, and these were all done within the first 1-2 weeks of therapy.

*Several months later the patient developed progressive anemia and leucocytosis. Died seven months later.

been advocated in other clinics (see footnote, page 110). In spite of this, the erythrocyte count after therapy fell below 4,000,000 cells per cubic millimeter in eleven of the thirty patients. In most of the other reports in the literature, the patients had not been followed for as long a period of time, or the data presented were insufficient to determine whether or not the patients were over-treated. However, as has already been emphasized, similar transient anemias were noted in patients treated by Erf and Jones¹⁴ and by Hall and co-workers.¹⁶

The figures in column 5 reveal that in our cases the erythrocyte level did not begin to fall until three to six weeks after the initial injection was given. The

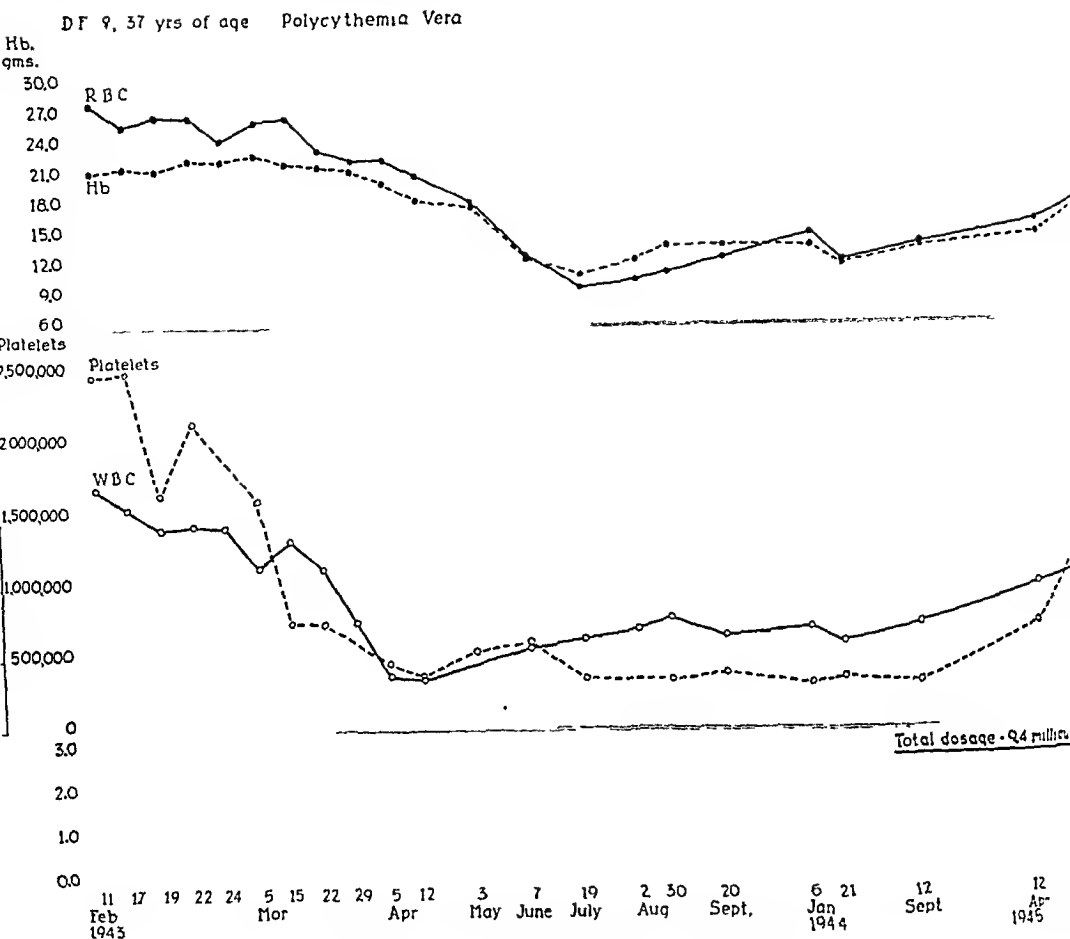


Fig. 4.—Polycythemia vera. Response to therapy with P³².

Case Summary.—D. F., a housewife, 37 years of age, had had frequent dull headaches, often accompanied by dizzy spells, since 1935. She also complained of deep aching pains in the thighs and legs. Itching of the skin was severe at night and after bathing. During 1942 the headaches and dizziness increased in frequency and severity, and she began to tire easily. She had been “nervous” for many years.

Physical Examination (Feb. 11, 1943): Complexion was ruddy and there was marked cyanosis. The conjunctivae were injected. The heart and lungs were normal, and the blood pressure was 140/90. The spleen extended 6 cm. below the costal margin.

Laboratory Data: Blood counts (Feb. 11, 1943): erythrocytes, 9,360,000; hemoglobin, 21.3 grams; leucocytes, 16,850; reticulocytes, 2.8 per cent; platelets, 2,460,000; differential, normal. The total blood volume was 11,080 c.c. (173 c.c. per kilogram of body weight). Urinalysis, negative. The Kahn reaction was negative. The venous pressure and circulation time were normal. An electrocardiogram was normal except for left axis deviation. X-ray films of the chest and of the skull revealed no significant abnormalities.

Course: About a month after P³² therapy was started the patient began to feel better. This symptomatic improvement continued and within a few months she had no complaints except an occasional mild headache, rare episodes of pruritus, and spells of weakness and dizziness just before the menstrual periods. She continued to feel well until about September,

(Continued on opposite page.)

interval was much longer than this in some cases, but these were patients who received a relatively small initial dose or else lived so far from St. Louis that they could be followed only at infrequent intervals.

Erf's explanation¹⁴ for this long latent period between the first injection of P^{32} and the first significant fall in the erythrocyte level certainly seems reasonable. He suggested that since human red blood cells apparently circulate for from 90 to 120 days, the effect on the total count of a slower rate of red cell delivery from the marrow would not become apparent for several weeks. This delayed fall in the erythrocyte level has several obvious practical disadvantages. If a patient's symptoms are severe, or the hematocrit value so high that some complication such as cerebral thrombosis or myocardial infarction is feared, it may not be possible or wise to wait for the full effect of P^{32} to become evident. These circumstances were encountered in three of our thirty patients, and on them phlebotomies were done; 500 cubic centimeters of blood were removed per day and no subject was bled more than four times. It is probable that this procedure would have been used more frequently during the first two weeks of P^{32} therapy if a determined effort had not been made to keep treatment as uncomplicated as possible.

As has been pointed out in previous publications,^{16, 19} therapy with P^{32} as ordinarily employed in the treatment of polycythemia vera not only affects the erythrocyte level, but also has a profound effect on the platelet and leucocyte counts. The average platelet count of our polycythemic patients was 1,760,000 per cubic millimeter before therapy; this fell to an average minimum of 367,000 per cubic millimeter after the first course of treatment. In only one instance did the platelets fall below 50,000 per cubic millimeter. The average leucocyte count before treatment was 13,250 per cubic millimeter; after the first course of treatment, the average minimum count was 4,400 per cubic millimeter, but values as low as 950 were observed. In most cases in which a severe thrombocytopenia or leucopenia developed, this phase lasted only a few weeks. However, in general, the platelet and leucocyte levels have not returned to their original high values.

That the response to therapy varied greatly in different patients is apparent from Table 2. Patient 8 received 5.66 millicuries of P^{32} and the erythrocyte count dropped from 8,110,000 to 2,970,000 cells per cubic millimeter, whereas Patient 9 received 6.71 millicuries and the erythrocyte level dropped only from 7,910,000 to 4,770,000 cells per cubic millimeter. There are many other equally striking discrepancies in the hematologic response observed in different patients to a given dosage. *Consequently, therapy must be individualized to a high degree, and it is impossible to predict in advance how much therapy any given patient will require.*

1943, when she began to have severe hot flashes and increasing nervousness. Considerable illness among members of her family, the death of her father, domestic difficulties, and the fact that the patient was working as a clerk in a store, in addition to taking care of her home, tended to aggravate her symptoms. In general, during the following two years she felt considerably better than she had felt prior to P^{32} therapy. However, she continued to have hot flashes, nervousness, fatigability, and numerous minor complaints, all of which were thought to be on either a menopausal or psychogenic basis. Stilbestrol relieved some of these complaints temporarily.

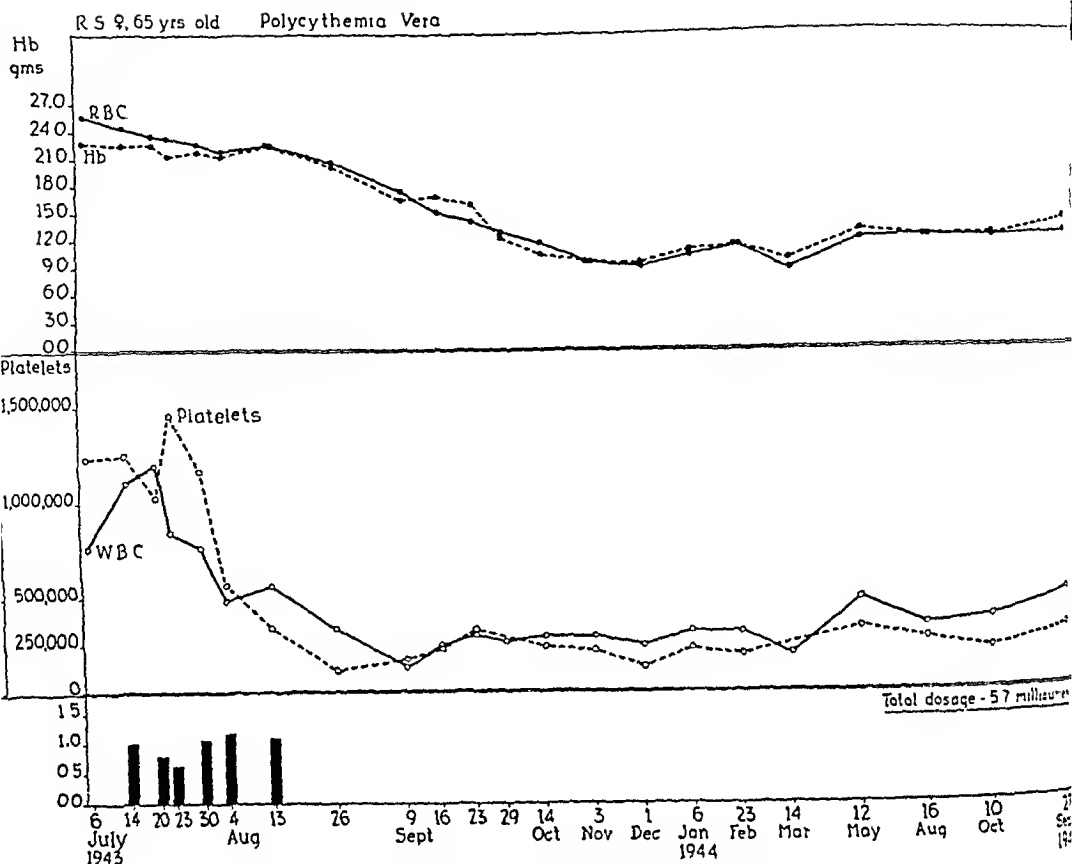


Fig. 5.—Polycythemia vera. Response to therapy with P^{32} .

Case Summary.—R. S., a widow, 65 years of age, suddenly developed numbness in the left hand in January, 1940; within a week this had progressed and involved the whole arm. Shortly thereafter she began to have precordial aching pains, and occasional aching pain in her left shoulder. In January, 1941, she had pain in the left upper quadrant for the first time; her physician told her this was due to an enlarged spleen. About July, 1941, she began to tire very easily and felt drowsy much of the time. These symptoms persisted with little change until May, 1943, when she began to have severe pain in her feet, especially the toes, at night. By this time she had difficulty using her left hand because of numbness and stiffness. Itching after bathing was intense. Her weight decreased by nineteen pounds in two years' time.

Physical Examination: Blood pressure was 185/115. The complexion was very ruddy, and there was some cyanosis of the lips. Lymph nodes were not enlarged. The heart was enlarged. The spleen edge measured 18 cm. below the costal margin, and the liver edge 4 cm.

Laboratory Data: Blood counts (July 14, 1943): erythrocytes, 8,110,000; hemoglobin, 22.3 Gm.; leucocytes, 11,100; reticulocytes, 1.8 per cent; platelets, 1,230,000; differential: polymorphonuclear neutrophils, 73 per cent; stabs, 13 per cent; juveniles, 2 per cent; basophiles, 1 per cent; lymphocytes, 5 per cent; monocytes, 6 per cent. The sternal bone marrow was extremely hyperplastic; the clumps of marrow were so dense that it was impossible to do an accurate count. Erythroid stimulation was marked, and myeloid cells and megakaryocytes were also present in increased numbers. Urinalysis was negative. The Kahn reaction was negative. Blood indices, coagulation time, and bleeding time were nor-

(Continued on opposite page.)

Detailed data for two patients are graphically illustrated in Figs. 4 and 5.

2. Effect of Therapy on the Clinical Manifestations of Polycythemia Vera:

The clinical manifestations shown by these thirty patients, both before P^{32} was given and after the maximum benefit from therapy had been obtained, are summarized in Appendix Table 1. In every case, the patient felt greatly improved, at least temporarily, following therapy. However, in only eight patients were all symptoms completely relieved. This is probably attributable to the fact that many of the patients had disorders such as arteriosclerosis, hypertension, arthritis, menopausal syndrome, or psychoneurosis which accounted for some of their symptoms. Furthermore, the symptoms after therapy in many cases were much less troublesome than they formerly had been. In Table 3 are listed the more common symptoms of polycythemia and the percentage of our patients in whom each symptom was partially or completely relieved. It will be noted that headache is the only symptom which was not completely relieved in over 50 per cent of the patients. Of the 64 per cent of patients who still had headaches after therapy, the majority said their headaches had become mild and infrequent, and they mentioned them only when specifically questioned.

TABLE 3. THIRTY PATIENTS WITH POLYCYTHEMIA VERA—EFFECT OF P^{32} THERAPY ON SYMPTOMS

	NUMBER OF PATIENTS WITH SYMPTOM	% OF PATIENTS COMPLETELY RELIEVED	% OF PATIENTS PARTIALLY RELIEVED	% OF PATIENTS UNIMPROVED
Fatigability	20	65	30	5
Headaches	22	36	64	0
Dizziness	15	53	47	0
Aching pains in extremities	14	57	36	7
Itching	15	53	40	7
Blurring of vision	11	73	18	9
Burning of eyes and/or lacrimation	5	100	0	0

In Table 4 are recorded the abnormal physical findings encountered in these patients with polycythemia vera and the percentage of patients in whom there was a complete or partial disappearance of the abnormality following therapy with radioactive phosphorus. If the spleen was palpable, it was considered to be enlarged and is recorded as "splenomegaly"; if, after treatment, the spleen was no longer palpable, it is recorded as "abnormal finding completely dis-

mal. The total blood volume was 7,780 c.c. The basal metabolic rate was +15 per cent. An electrocardiogram showed right bundle branch block and auricular premature contractions. An x-ray of the chest revealed only moderate cardiac enlargement and aortic lengthening. An x-ray of the cervical spine revealed extensive osteoarthritic changes.

Course: About six weeks after P^{32} therapy was started the patient's strength began to improve and the itching cleared up entirely. The aching pains in the hands, shoulders, and over the precordium persisted. Six months later she had no symptoms except the pains (which were thought to be due to arthritis), and even these had lessened in intensity. She tired much less easily and was more alert. The spleen had decreased in size and now measured 9 cm. below the costal margin; the blood pressure had fallen to between 150/80 and 175/90. On Sept. 27, 1945, twenty-six months after the first treatment, she felt well except for aching pain in both hands and in the left shoulder. She had gained twenty-seven pounds in weight. The spleen measured 4 cm. below the costal margin.

TABLE 4. THIRTY PATIENTS WITH POLYCYTHEMIA VERA—CHANGES IN PHYSIOAL SIGNS FOLLOWING P³² THERAPY

	NUMBER OF PATIENTS WHO HAD ABNORMAL PHYSICAL SIGN AT ONSET OF THERAPY	% OF PATIENTS IN WHOM AB- NORMAL FINDING COMPLETELY DISAPPEARED AFTER THERAPY	% OF PATIENTS IN WHOM ABNORMAL FINDING PARTIALLY SUBSIDED	% OF PATIENTS IN WHOM THERE WAS NO IMPROVEMENT
Skin and/or mucous membranes bright red	25	96	4	0
Cyanosis	15	93	7	0
Conjunctivae red	9	100	0	0
Retinal venules distended	8	100	0	0
Petechiae and/or ecchymoses	2	100	0	0
Splenomegaly	28	68	32	0
Hepatomegaly	8	62.5	37.5	0
Systolic blood pressure 150 mm. Hg or higher	11	36.5	36.5	27

appeared," even though it is recognized that the spleen may never have returned to a perfectly normal size. Hepatomegaly is arbitrarily defined as that condition in which a liver edge was felt 2 cm. or more below the costal margin in the mid-clavicular line. No patient who had hepatomegaly was recorded as showing a complete disappearance of the abnormal finding after therapy unless the liver edge was no longer palpable after therapy. In all patients who had hepatomegaly or splenomegaly, measurements were routinely recorded with the patient breathing as quietly as possible. The measurements were taken from the costal margin to the lower border of the spleen or liver in the midclavicular line and in the anterior axillary line; in addition, the distance from the medial border of the spleen to the right or the left of the mid-line was recorded. If the spleen was only moderately enlarged, the only measurement recorded was the distance from the tip of the spleen to the costal margin at the maximum point. From these standardized measurements it was possible to follow changes in the size of these organs with reasonable accuracy.

The bright red color of the skin and mucous membranes, the cyanosis, the reddened conjunctivae, and the distended retinal venules disappeared after treatment in practically all cases. The only patient in whom the bright red color and the cyanosis did not completely disappear was a patient who was followed for only three months after therapy and his erythrocyte count had never dropped below 6,300,000 cells per cubic millimeter. Splenomegaly was present in all but two subjects, whereas hepatomegaly was present only eight times. All of the patients who had hepatomegaly also had a rather marked splenomegaly, the spleen edge measuring from 2 to 18 centimeters below the costal margin. About two-thirds of the enlarged spleens and two-thirds of the enlarged livers became nonpalpable after treatment.

It is interesting that eleven of the thirty patients had a systolic blood pressure of 150 or more millimeters of mercury, and eight of these eleven had a diastolic pressure of 100 or more millimeters of mercury. Of the abnormal physical findings, hypertension responded least favorably to P³² therapy.

3. *Duration of Remissions Produced:* Twenty-one of the thirty patients (Patients 1 to 5 and 7 to 22 in Table 2) have been followed for a year or more. In them, the duration of remissions has arbitrarily been defined as the interval from the time the red blood cell count fell below 5.5 million cells to that at which it again rose above the 6.0 million level. The longest remission so far observed is thirty-three months and is still continuing. In five patients, the remissions have been longer than two years; in eleven, longer than one year; and in seventeen, longer than nine months. Most of these subjects have not yet required a second course of therapy (last column, Fig. 2) so that no average figures can now be calculated. Additional radioactive phosphorus, as a matter of fact, has been given to only eight of the twenty-one patients. In seven instances, the duration of remissions was shorter than twelve months. One patient (Patient 2, Fig. 2), after a short remission of only three months, developed a progressive anemia, leucocytosis, and a leucocyte differential indistinguishable from that of subacute myelogenous leukemia. At the time of her death, more than 50 per cent of the leucocytes were myeloblasts or early myelocytes.

C. *Comparison of Radioactive Phosphorus With Other Forms of Therapy Used in the Treatment of Polycythemia Vera.*—Radioactive phosphorus unquestionably induces both clinical and hematologic remissions, often of long duration, in patients with polycythemia vera. Similar effects have been obtained with x-ray therapy or phenylhydrazine, and many clinicians have found therapeutic phlebotomy a satisfactory method of treatment. There is a growing conviction, however, which is shared by us, that administration of radioactive phosphorus is the treatment of choice for polycythemia vera. There can be no doubt that it is the most convenient method for the patient. He experiences no radiation sickness, does not have to take a drug whose dosage must constantly be regulated with care, does not have to subject himself to the inconvenience of repeated phlebotomies. Radioactive phosphorus exerts its therapeutic effect, as does x-radiation, by slowing the rate of erythrocytogenesis. Long clinical and hematologic remissions may be produced by one course of therapy.

It is not yet possible to conclude, however, that radioactive phosphorus prolongs life to a greater degree than do other forms of therapy. Polycythemia vera is a chronic disease of long duration in which spontaneous remissions not infrequently occur. The chronicity of the disease is well illustrated by the cases studied at the Mayo Clinic.⁴⁷ When Tinney, Hall, and Giffin⁴⁷ reviewed their experience with 163 patients seen prior to November, 1941, they found that in thirty-six the disease was of five or more years' duration. In nineteen patients, polycythemia had been present for ten or more years; in eight, for at least fifteen years; and in four, for at least twenty years. Death had occurred in twelve of these thirty-six patients from the following causes: myelogenous leukemia or leukemoid reaction, six; hemorrhage, three; thrombosis of the superior mesenteric vein, one; carcinoma of the stomach, one; and cirrhosis of the liver with Paget's disease of the skull, one. The most common cause of death among these patients, therefore, was myelogenous leukemia (or an anemia associated with a leukemoid reaction). The fact that one of the individuals treated by Hall and his associates¹⁶ and one of our patients (Patient 2, Table 2) have

already died with similar manifestations suggests that radioactive phosphorus will not prevent this complication of polycythemia vera. An additional fifteen or twenty years of experience will be required before conclusions can be reached as to whether P^{32} will prolong life more than have x-ray therapy, phenylhydrazine, and phlebotomy.

Even though a number of the remissions obtained by other investigators and by us are now measurable in years and are still in progress, there is little reason to believe that cures have been produced in these instances. The number of observed relapses is great enough to make one believe that relapses will also eventually occur in these persons.

Emphasis has already been given to the fact that complications may result from therapy with P^{32} as from other forms of treatment. These are anemia, leucopenia, thrombocytopenia, and possibly aplastic anemia. Prevention of these complications can be accomplished at the present time only by avoiding overdosage. Because of difference in susceptibility of patients to the isotope, the dosage must be individualized to a high degree. The chief practical disadvantages associated with the use of radioactive phosphorus at the present time are (1) the moderately high cost of producing it, (2) its relative scarcity, and (3) the relatively long latent period (from thirty to sixty days) which occurs before the red blood cell count begins to fall.

D. Summary and Recommended Method for Administering P^{32} to Patients With Polycythemia Vera.—From the results reported in the literature and from those here recorded, it is clear that the administration of radioactive phosphorus to patients with polycythemia vera induces satisfactory clinical and hematologic remissions which not infrequently last for two or more years. The fall in the red blood cell count, however, usually does not begin for from thirty to sixty days; for this reason, if symptoms are severe, it is often advisable to reduce the red cell level by removing blood during this period. Treatment with P^{32} seems superior to other methods used in the past, but it is not yet certain that this form of therapy will prolong life to a greater degree than will x-radiation, phenylhydrazine, or repeated phlebotomies. Dosage must be individualized and carefully controlled if the production of anemia, leucopenia, and thrombocytopenia is to be avoided.

As a result of the experience accumulated in this clinic, radioactive phosphorus is now given to patients with polycythemia vera according to the following routine. After the diagnosis has been established, from 3.5 to 4.0 millieuries are injected intravenously. The height of the red blood cell count and the weight of the patient are used as a guide to decide which of these doses will be employed (see footnote, page 110). No additional P^{32} is given for three months. If symptoms are severe, however, or the erythrocyte level is so high that complications such as thrombosis are feared, 500 cubic centimeters of blood are removed daily until the red cell count has been reduced to between 6 and 6.5 million cells. This level is maintained by additional bleedings during the first two to four weeks. They are then discontinued, if possible, so that the effectiveness of the P^{32} may more easily be evaluated. If the count remains above 6.0

million cells, a second injection of from 1.0 to 3.0 millicuries is given about ninety days after the first injection. Additional phlebotomies are usually not required but are done if the erythrocyte level rises high enough so that symptoms reappear. Many patients do not require this second injection; a few must be given a third injection of from 1.0 to 3.0 millicuries after a second interval of ninety days. By this time a remission has almost invariably been produced and the patient is observed at intervals of from one to two months either by us or by his own physician. No additional therapy is given until a relapse occurs. This schedule is more conservative than that used by most other clinicians who have used radioactive phosphorus therapeutically but has been adopted because it rarely causes any of the cellular elements of the blood to fall significantly below normal levels.

IV. MYELOGENOUS LEUKEMIA

A. Review of the Literature.—There are nine reports in the literature dealing with the therapeutic effectiveness of radioactive phosphorus in myelogenous leukemia.^{1-3, 5, 7, 9, 10, 12, 15} These reports are based on the study of 107 patients of whom eighty-two had chronic myelogenous leukemia, twenty had acute myelogenous leukemia, and five had subacute myelogenous leukemia.

Lawrence¹ presented detailed data on two patients. The first was a 52-year-old man with chronic myelogenous leukemia. This man was given 86 millicuries of radioactive phosphorus by month during a period of seventy-two days. Throughout this time there was progressive symptomatic improvement. The total leucocyte count dropped from 600,000 cells per cubic millimeter to within the normal range; all myeloblasts and myelocytes disappeared from the blood; and the erythrocytes rose from 3,350,000 cells per cubic millimeter to normal values. There was a gradual and progressive decrease in the size of the liver and spleen until neither was palpable. The patient remained symptom-free for a period of about four months, at the end of which time the leucocyte, erythrocyte, and thrombocyte levels all decreased to low levels and hemorrhagic manifestations appeared. Shortly thereafter, the leucocyte count rose progressively, and the liver and spleen enlarged. Severe cough, fever, and night sweats developed, and cellulitis of the face occurred terminally. At post-mortem examination, miliary tuberculosis was found; there was a mild amount of myeloid hyperplasia in the bone marrow and slight myeloid metaplasia in the spleen and liver.

The second patient was a woman, 40 years of age, who had acute myelogenous leukemia. She was given iron, vitamins, liver extracts, and small doses of x-ray therapy; the total leucocyte count dropped temporarily following each x-ray treatment, but there was no clinical improvement. The patient was then given two oral doses of radioactive phosphorus four weeks apart (4.77 and 1.19 millicuries, respectively). The leucocyte count fell to leucopenic levels, but again there was no clinical improvement. At death, post-mortem examination revealed pathologic changes of acute myelogenous leukemia without histologic evidence of irradiation effects.

Since this original report by Lawrence, specific data have been published on fifty-three additional cases. Forty-three previously unreported cases were analyzed in a comprehensive paper by Erf, Tuttle, and Lawrence.⁵ It is obviously impossible to summarize each of these cases here, and only their general conclusions will be discussed. All of the other detailed case reports in the literature, ten in number, are summarized in Table 5.

Erf, Tuttle, and Lawrence⁵ tabulated the symptoms and physical signs before and after P₃₂ therapy of thirty-eight patients with chronic myelogenous leukemia and eight patients with acute myelogenous leukemia. The total dosage of radioactive phosphorus given to the patients with chronic myelogenous leukemia ranged from 2.3 to 90.7 millicuries, with an average total dosage of 26.5 millicuries. Among the patients with acute myelogenous leukemia, the total dosage varied from 2.0 to 24.1 millicuries, with an average of 10.4. The hematologic

TABLE 5. SUMMARY OF REPORTS FROM THE LITERATURE ON PATIENTS WITH MYELOGENOUS LEUKEMIA TREATED WITH P₃₂

AUTHOR AND REFERENCE	PATIENT	AGE AND SEX	APPROX. INITIAL W.B.C. COUNT (PER C.M.M.)	TREATMENT	COMMENTS
Warren ²	1	35, M	41,200 (50 per cent "blasts")	4 injections totaling 5.9 mc. over period of 6 weeks; 4 blood transfusions also given	Subacute myelogenous leukemia; during period of therapy W.B.C. fluctuated widely, rising terminally to 97,500 cells per c.mm. with more than 70% blasts; patient died about 44 days after start of P ₃₂ therapy
Lawrence ³	1	58, F	22,000	5 doses given orally over period of 10½ months (total, 16.22 mc.)	Each treatment was followed by restoration of approximately normal leucocyte level and disappearance of leukemic skin rash; patient still living and in excellent clinical condition 13 months after onset of therapy
	2	29, M	132,000	3 doses given orally over period of 7 months (total, 10.74 mc.)	Following treatment, leucocyte count gradually dropped and erythrocyte level rose to approximately normal; shortly after first dose of P ₃₂ he improved clinically and 1 year later was still symptom-free; liver and spleen, which were initially greatly enlarged, became no longer palpable
	3	39, F	155,000	5 doses given orally over period of 2 months (total, 21.00 mc.)	Following therapy leucocyte count dropped to 8,000 and then fluctuated between 10,000 and 20,000 for next 6 months; erythrocyte count rose to normal; splenomegaly and hepatomegaly disappeared
Kenney ⁹	1	25, F	307,200	8 doses (? route) totaling 18.9 mc. over period of 5½ weeks	3 months after start of P ₃₂ therapy W.B.C. count was 12,000 and spleen had decreased markedly in size; 1 month later W.B.C. count began to rise rapidly; this continued in spite of x-ray therapy; died 4½ months after onset of P ₃₂ therapy
	2	41, M	310,000	23 doses (? route) totaling 50.8 mc. over period of 15 months	Leucocyte count fairly well controlled by treatment; erythrocyte level rose from 3,400,000 to around 4,500,000; patient still living at time of report
Low-Beer and associates ¹²	1	12, F	380,500	14 doses totaling 16.038 mc. given over period of 12 weeks; 3 doses (about 1 mc. each) given during next 5 months; additional 7 doses totaling 4.13 mc. during last 6 weeks of life	14 weeks after onset of P ₃₂ therapy W.B.C. count was 10,300, R.B.C. had risen from 2,070,000 to 5,180,000, spleen, which originally filled most of the abdomen, was barely palpable; patient gained 20 pounds, felt well; fairly complete remission lasted about 4 months, but death occurred 10 months after onset of therapy

TABLE 5—CONT'D

AUTHOR AND REFERENCE	PA- TIENT	AGE AND SEX	APPROX. INITIAL W.B.C. COUNT (PER C.M.M.)	TREATMENT	COMMENTS
Low-Beer and asso- ciates ¹²	2	44, F	342,400	P ³² given orally; exact dosage not given, but radia- tion level was built up to a 5.0 mc. level	Within 6 weeks leucocyte count dropped to normal and erythro- cyte count rose to normal; pre- viously palpable lymph nodes disappeared and patient gained 10 pounds; patient remained in excellent condition with very slight fluctuations of blood counts for 8 months; two sub- sequent relapses were treated with courses of P ³² therapy; at time case was reported, patient was again in excellent remis- sion (11 months after onset of therapy)
Warren ¹⁵	1	45, F	322,000	8 i.v. injections to- taling 20.41 mc. given between 11/20/40 and 4/6/42	Patient improved clinically and spleen became smaller; follow- ing initial drop in W.B.C. count, leucocyte level fluctuated be- tween 14,400 and 44,000 until terminally, when it rose to 320,000; during first few months of therapy, R.B.C. count rose slightly; patient expired 17 months after onset of therapy
	2	60, F	250,000	14 doses (all but 4 i.v.) totaling 29.62 mc. be- tween 4/17/42 and 6/4/43	This patient had failed to respond to x-ray therapy and was virtu- ally moribund when P ³² was started; she improved clinically and spleen decreased to two- thirds its former size; W.B.C. count never dropped below 80,000; death occurred 19 months after onset of therapy

changes during and after treatment were briefly summarized for ten of the patients with chronic myelogenous leukemia. Apparently, three of these had previously been reported by Lawrence and his associates. None of the patients with acute leukemia was benefited by therapy, and all were dead at the time of the report. Of the thirty-eight chronic cases, eleven of the patients had partial remissions and five had complete remissions; twenty-one had died. Two patients had essentially complete remissions which were of nearly two years' duration at the time of the analysis. Of the thirty-eight chronic cases, twelve of the patients had had no therapy previous to P³². The response to P³² was less satisfactory in those who had had x-radiation previously. As the duration of the disease previous to the administration of P³² was quite similar in the two groups, these investigators concluded that previous x-radiation reduces the effectiveness of radiophosphorus. In those cases examined at autopsy, the gross findings were those usually described for leukemia. The histopathology was interesting in that there were no cellular changes in normal cells that could be attributed to local irradiation caused by the radiophosphorus; many sections did show cellular lysis or rhexis in leukemic cells. The authors concluded that in the doses they employed, radiophosphorus produced no morphologic damage to normal tissues.

Craver⁷ summarized the results obtained in the treatment of eleven adults with chronic myelogenous leukemia. No specific data concerning the dosage of P³² employed or changes in the blood counts were given for any of these patients. Only one was followed for as long as one year. Three of the eleven patients died 20.5, 4, and 4 months, respectively, following the

beginning of P^{32} therapy, whereas eight were still living at the time of the report. Craver concluded that of the eleven patients, "seven, including the one who died after 20.5 months, showed definite evidence that P^{32} is an effective therapeutic agent in this type of leukemia." Craver also mentions that one patient with acute myelogenous leukemia and one with subacute myelogenous leukemia were treated. One of these died in one week and the other in six weeks; in neither patient was there any detectable effect of P^{32} , favorable or unfavorable. No details were given.

Kenney⁹ reported having treated six patients with chronic myelogenous leukemia, and one each with acute and subacute myelogenous leukemia. Case reports were given for only two patients; data for these two are summarized in Table 5. The patient in the acute phase of the disease died one week after the administration of 1.5 millieuries of P^{32} . The patient who was in the subacute phase of the disease received three doses of 0.8 millieuries each at weekly intervals; death occurred six weeks after the first treatment. In all six individuals with chronic myelogenous leukemia, the following effects were observed: the leucocyte count was reduced to approximately normal levels; the percentage of myeloblasts and myelocytes in the bone marrow was reduced; the erythrocyte counts and hemoglobin levels rose during therapy; enlarged spleens decreased in size; and in no case was radiation sickness observed.

Fitz-Hugh and Hedes¹⁰ reported having treated five patients with chronic myelogenous leukemia, but no data were given concerning any of them. The authors state that one patient obtained an excellent remission which lasted several months, and a second patient who was then under treatment seemed to be responding satisfactorily. The other three persons were in the late stages of the disease and were unimproved by treatment.

Nineteen patients with chronic myelogenous leukemia, two with subacute myelogenous leukemia, and nine with acute myelogenous leukemia were treated with radioactive phosphorus by Warren.¹⁵ Only two case reports were given. These are summarized in Table 5. Warren stated that nine of the nineteen patients with chronic myelogenous leukemia were "helped" by therapy, both cases of subacute myelogenous leukemia were "helped," but none of the acute cases was benefited. These patients were considered helped who "showed definite clinical and laboratory evidence of improvement."

There is substantial agreement among all these investigators that patients with acute myelogenous leukemia do not respond favorably to therapy with P^{32} . There is also agreement that in chronic myelogenous leukemia administration of radioactive phosphorus will usually restore the leucocyte count to normal or approximately normal levels, and that a rise in the erythrocyte level frequently follows. This hematologic improvement is quite consistently accompanied by definite symptomatic improvement. As in polycythemia vera, adequate data are given for so few cases of myelogenous leukemia that it is not possible to evaluate the duration of the remissions induced or to compare the results of P^{32} therapy with the results which have been obtained with other forms of treatment such as x-ray. Detailed data are given in the following section for all of our patients in the hope that they may provide at least tentative answers to these important problems.

B. Analysis of Results Obtained in Treating Thirty-Nine Patients With Myelogenous Leukemia.—Thirty-nine patients with myelogenous leukemia have been treated with radioactive phosphorus at the Edward Mallinckrodt Institute of Radiology. Twenty-one of these have died, and eighteen are still living. During the first ten days to two weeks of therapy, each patient was seen once every two or three days. Thereafter, the interval was gradually lengthened as the disease was brought under control, but even during protracted intervals of complete freedom from symptoms, each patient was seen at least once every two months. A sternal bone marrow examination was done in every case immediately before therapy was begun and at variable intervals thereafter depending on the severity and progress of the disease. A total of from two to four sternal aspirations have been performed on most of the patients who have been

under observation for two years or longer. The basal metabolic rate was also followed in most instances.

It is important to emphasize that there was no selection of patients for this series. Only those who lived so far away that they could not be seen at frequent intervals were rejected and treated with x-radiation or Fowler's solution. Many of the thirty-nine persons to whom P^{32} was given had been treated for years by their family physicians and were referred to us only when previously employed therapeutic measures no longer seemed effective. The average duration of symptoms at the time P^{32} therapy was started was slightly over two years, and ten of the patients had had symptoms for three years or longer. At least nine patients (Patients 1, 6, 16, 19, 20, 21, 25, 28, and 38) were either considered to have subacute myelogenous leukemia or to be in the terminal stage of chronic myelogenous leukemia.

1. *Effect of Therapy on the Cellular Elements of the Blood:* The hematologic and various other data on these patients are summarized in Table 6. Twenty-one of the subjects were men, and eighteen were women. Twenty-four of them had had some other form of treatment before P^{32} was first administered, including x-radiation in twenty cases. In very few instances, however, had any other therapy been given during the last four months prior to the first injection of P^{32} . The initial leucocyte count was elevated in all thirty-nine patients, and there were no examples of subleukemic leukemia in this series. Thirty-four of the thirty-nine patients had an anemia of less than 4,000,000 cells per cubic millimeter, and seven had a thrombocytopenia of less than 300,000 platelets per cubic millimeter (this is definitely below the normal range by Dameshek's method).

The wide variation in the dosage of radioactive phosphorus required to restore the leucocyte count to approximately normal levels needs to be emphasized. In eight patients less than 6 millieuries sufficed to reduce the white blood cell count to 15,000 or less, while two patients received in excess of 50 millieuries without ever showing a drop of the leucocyte count to this level. In nineteen of the patients the dosage required to lower the leucocyte count to less than 15,000 cells per cubic millimeter was between 6 and 12 millieuries; the time required varied from 15 to 120 days. The height of the initial leucocyte count was not the only determining factor for either the total amount of P^{32} or the time required to accomplish this result.

As the leucocyte count decreased, the percentage of polymorphonuclear leucocytes increased and the percentage of myelocytes and myeloblasts decreased significantly in every case except one. This one patient (Patient 25) was in the terminal stage of the disease and died nine days after treatment was begun. The erythrocyte level increased by more than half a million cells per cubic millimeter in twenty-five of the thirty-three patients who received no transfusions; in twelve instances the increase amounted to more than 1,000,000 cells per cubic millimeter. The platelet count was less than 300,000 per cubic millimeter at the time P^{32} therapy was started in seven patients; in four, the thrombocyte level rose after treatment to above 900,000 per cubic millimeter. Bone marrow examinations were usually repeated after the blood counts had been

TABLE 6. HEMATOLOGIC DATA ON THIRY

PATIENT	AGE AND SEX	PREVIOUS TREATMENTS	BLOOD COUNTS AT TIME P32 THERAPY WAS STARTED						P32 THERAPY REQUIRED TO RESTORE W.B.C. COUNT TO APPROX. NORMAL (4,000-15,000)		MOST NORMAL BLOOD COUNT AFTER P32 THERAPY				
			W.B.C. (PER C.M.M.)	R.B.C. (MILLIONS PER C.M.M.)	HG (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% POLYMORPHONUCLEAR NEUTROPHILES	% MYELOCYTES + MYELOBLASTS	MC.	DAYS	W.B.C.	R.B.C.b	HG (GM.)	PLATELETS	% POLYMORPHONUC.
1	38, M	X-R	135,000	3.59	11.6	164,000	1	43	19.744	69	11,000	4.44	14.3	1,830,000	4
2	25, F	Fo	155,000	3.69	10.8	1,180,000	17	33	9.537	33	8,700	4.45	12.5	1,436,000	8
3	58, M	X-R	111,000	5.51	15.1	1,664,000	26	23	9.704	47	8,100	5.42	14.7	1,471,000	7
4	42, M	X-R, Fo	72,000	3.26	8.7	593,000	14	11	7.415	18	7,850	3.56	8.9	1,120,000	5
5	39, F	X-R	107,000	3.78	12.3	3,553,000	30	29	4.712	24	8,500	4.64	13.5	3,544,000	7
6	41, M	None	497,000	2.52	7.4	25,000	27.5	62.5	1.251	23	7,150	4.31 T-1	10.9	1,100,000	6
7	67, F	X-R	464,000	2.25	7.3	46,000	15	44	5.780	63	7,600	4.63	13.0	1,150,000	7
8	50, F	X-R, Fo	115,000	2.35	7.5	921,000	35	28	9.589	37	7,500	3.14 T-3	9.6	1,320,000	7
9	57, M	X-R	22,000	5.05	16.5	1,179,000	43	10	3.470	22	7,400	5.61	17.5	1,390,000	7
10	37, M	X-R, Fo, T.	144,000	3.88	9.4	2,663,000	40	34	69.863c	539	16,300	5.10	11.0	1,460,000	4
11	43, F	None	344,000	2.96	8.6	444,000	21	29	16.838	119	8,750	4.43	12.9	1,287,000	6
12	58, F	X-R	288,000	2.83	6.5	1,560,000	3	52	25.324	190	8,050	3.57 T-4	9.0	1,150,000	7
13	48, F	X-R	164,000	2.90	7.9	487,000	12	37	20.128	124	7,850	4.44	12.5	1,509,000	7
14	37, F	None	300,000	2.94	8.0	1,117,000	18	34	19.493	143	6,750	5.27	13.9	1,420,000	6
15	31, M	None	123,000	3.23	10.8	652,000	34	25	9.583	70	6,400	5.10	14.7	1,348,000	6
16	26, M	X-R	25,450	4.48	14.4	591,000	37	9	5.264	30	7,800	4.12	12.5	870,000	6
17	35, F	None	175,000	3.48	11.5	557,000	43	14	6.200	80	8,200	4.58	15.4	1,540,000	6
18	13, M	None	502,000	1.93	6.4	559,000	13	35	8.806	120	8,300	5.76	13.8	1,300,000	6
19	31, M	X-R	227,000	2.07	4.7	200,000	34	49	5.631	30	8,450	3.57 T-15	11.0	485,000	4
20	27, M	X-R	139,000	2.05	6.5	185,000	27	42	4.532c	9	89,000	2.62 T-3	6.3	225,000	3
21	52, M	X-R	429,000	2.08	6.2	360,000	6	50	13.956c	81	35,250	2.65	9.3	620,000	2
22	47, M	Fo	134,000	3.74	12.5	740,000	36	37	6.756	34	7,500	5.27	14.9	1,200,000	7
23	64, M	Fo, X-R	389,000	2.72	9.4	1,314,000	20	27	11.758	43	7,450	5.19 T-2	14.1	1,313,000	7
24	63, F	None	109,000	3.17	9.8	1,376,000	16	32	8.245	38	8,100	3.89	11.3	1,487,000	5
25	41, M	X-R	77,000	1.60	4.9	371,000	28	16	1.770c	3	56,500	1.08	3.9	194,000	2
26	42, M	X-R	53,000	6.14	15.1	602,000	50	8	5.063	17	8,900	5.67	15.4	1,531,000	8
27	16, M	Fo, B.M.	34,050	5.37	15.0	1,058,000	30	24	8.249	71	10,600	3.99	13.3	733,000	7
28	78, F	None	492,000	2.70	7.4	842,000	6	41	3.270c	5	443,000	1.46	4.4	510,000	2
29	51, F	None	243,000	3.32	7.0	1,477,000	14.5	44.5	7.315	30	8,000	5.00	14.2	1,540,000	4
30	36, F	None	550,000	2.10	6.8	270,000	7	45	54.590c	400	22,350	4.53	11.5	936,000	7
31	22, F	None	110,000	3.81	11.9	2,095,000	27	23	7.825	29	8,700	4.52	13.5	1,540,000	8
32	39, M	X-R	83,000	3.36	10.5	410,000	27	16	7.229	67	14,900	5.40	15.4	1,510,000	4
33	57, M	X-R, Fo	53,000	3.40	10.8	1,394,000	20	43	9.035	69	14,800	4.65	13.3	1,620,000	5
34	22, F	T	244,000	2.01	5.6	320,000	13	28	8.401	35	7,300	3.42 T-2	9.0	1,300,000	7
35	46, F	None	258,000	3.58	10.9	805,000	31	15	8.515	85	7,200	4.49	14.2	900,000	7
36	31, F	X-R	64,000	3.60	11.7	790,000	36	30	8.056	15	7,000	4.56	13.5	920,000	6
37	45, M	None	228,000	3.99	9.8	1,080,000	26	16	15.513c	132	20,450	4.75	13.8	1,050,000	6
38	76, M	None	56,500	1.96	6.4	85,000	23	37	4.256	10	6,250	2.64	7.3	120,000	6
39	38, F	None	132,000	3.42	9.2	1,265,000	23	31	6.59	47	10,950	4.76	13.0	1,590,000	8

*X-R = X-ray therapy; Fo = Fowler's solution; B.M. = bone marrow extract; T = transfusions.
 bT = Transfusions. The figure indicates the number of transfusions given.

CHRONIC MYELOGENOUS LEUKEMIA

TREATMENT AND BLOOD COUNTS DURING FIRST YEAR OR FRACTION THEREOF FOLLOWING RESTORATION OF LEUCOCYTE COUNT TO APPROXIMATELY NORMAL (4,000-15,000)		TREATMENT AND BLOOD COUNTS DURING SECOND YEAR OR FRACTION THEREOF FOLLOWING INITIAL RESTORATION OF LEUCOCYTE COUNT TO APPROX. NORMAL			TREATMENT AND BLOOD COUNTS DURING THIRD YEAR FOLLOW- ING INITIAL RESTORA- TION OF LEUCOCYTE COUNT TO NORMAL			DURATION OF DISEASE (MO.) FROM FIRST SYMPTOM	
W.B.C. RANGE	R.B.C. RANGE ^b	MC. OF P ₃₂	W.B.C. RANGE	R.B.C. RANGE ^b	MC. OF P ₃₂	W.B.C. RANGE	R.B.C. RANGE ^b	TO START P ₃₂	TO DEATH OR 9/1/45
11,100-358,000	4.44-1.09 T-3							18	26
1,650-152,000	4.45-2.33 T-6							48	59
1,600-22,650	3.56-5.66	1.654	10,350-19,450	4.49-5.80	None	9,800- 18,200	5.13- 5.95	15	54+
7,850-184,000	3.56-2.79							36	40
8,150-26,450	4.63-3.13	9.080	12,700-28,650	4.54-1.64 T-3				26	64
7,150-383,000	2.19-4.31 T-4							36	41
5,400-25,800	3.26-4.69	5.095	6,150-114,000	3.42-4.63	5.994	6,650- 31,800	3.93- 4.64	36	73+
3,800-17,500	1.62-3.14 T-13							60	63
6,400-15,150	4.11-6.20	2.150	9,700-15,600	5.15-7.14	2.088	13,250- 16,000	5.88- 6.76	27	64+
								39	59
8,750-150,000	4.43-1.38 T-15	2.564	2,500-48,500	0.87-2.42 T-8				9	31
1,550-32,550	3.57-1.32 T-6							11	30
7,850-221,000	4.44-2.27							54	66
6,550-48,000	2.05-5.76							30	44
6,400-21,000	3.62-5.10	11.991	11,800-30,350	4.05-5.10	1.026	24,200- 31,800	4.10- 3.84	6	34+
1,950-11,200	4.12-1.63							4	7
7,650-46,500	3.84-5.01	5.482	7,500-45,100	4.18-4.87				4	28+
8,300-120,000	5.76-2.30							18	27½
32,400-900	3.57-0.93 T-12							10	15½
								24	24½
3,500-67,500	5.27-3.69	4.960	7,500-65,000	4.32-4.98				21	24
1,850-51,500	2.80-5.19 T-1							24	42+
								60	72
8,100-60,500	3.89-1.96 T-6							12	20
7,800-55,500	4.76-6.10	6.235	12,250-47,000	5.32-5.58				62	62½
7,350-100,000	4.22-1.54 T-14							6	21+
								10	20
3,800-31,350	3.64-5.00	0.700	15,200-17,400	4.37-3.70				96	96½
								12	26+
5,800-14,000	3.70-4.52							12	25+
14,900-56,100	4.75-5.40							6	18+
14,000-43,300	1.65-3.03 T-9							6	17+
								20	31+
7,300-600	3.42-1.81 T-21							14	19+
2,400-10,000	4.10-4.60							24	30+
								2	7+
1,700-10,450	1.38-2.64 T-4							10	15+
								18	22+
								30	22+

are represents the total number of millicuries administered, but leucocyte count never returned to normal.
on x-ray therapy (300 r. units) terminally.

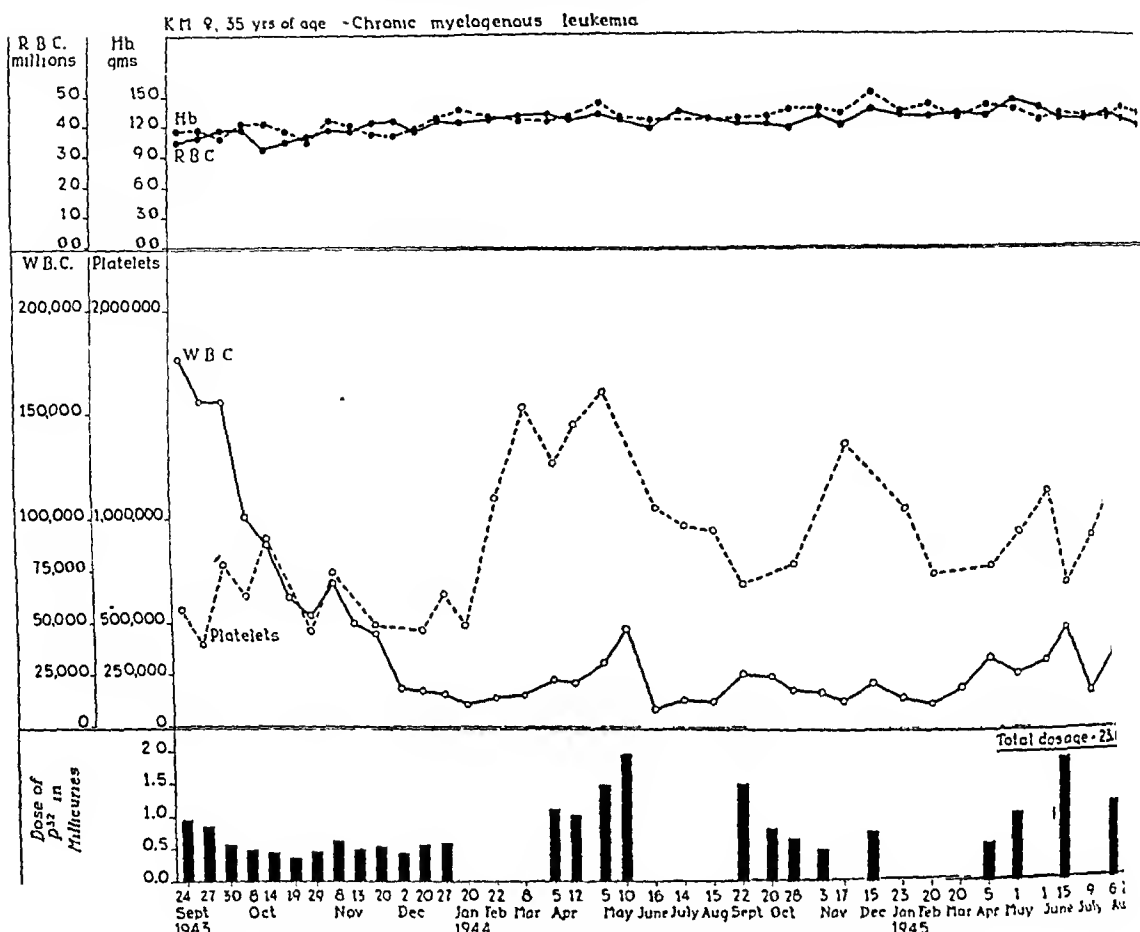


Fig. 6.—Chronic myelogenous leukemia. Response to P^{32} therapy.

Case Summary.—K. M., a housewife, 35 years of age, began to notice large spontaneous bruises scattered over the body in June, 1943. Shortly thereafter she noted mild fatigability. These symptoms persisted, but there were no other complaints. She had had severe rheumatic fever at the age of 12 and a heart murmur ever since that time.

Physical Examination: Patient was moderately obese. There were four bruises on the legs and three on the arms. There was one small hemorrhage in the left fundus. The heart was moderately enlarged, and a loud, harsh, systolic murmur was heard at the apex. The splenic edge measured 6 cm. below the costal margin in the mid-clavicular line. The liver was not enlarged. There were no palpable lymph nodes.

Laboratory Data: Blood counts (Sept. 24, 1943): leucocytes, 175,000; erythrocytes, 3,480,000; hemoglobin, 11.5 Gm.; reticulocytes, 1.0 per cent; platelets, 557,000; differential: segmented polymorphonuclear neutrophils, 43 per cent; stabs, 10 per cent; basophiles, 2 per cent; juveniles, 27 per cent; "C" myelocytes, 14 per cent; lymphocytes, 2 per cent; monocytes, 2 per cent. The sternal bone marrow on Sept. 24, 1943, was markedly hyperplastic, and the differential was: polymorphonuclear neutrophils, 23 per cent; stabs, 25 per cent; juveniles, 19 per cent; "C" myelocytes, 22 per cent; "B" myelocytes, 6 per cent; "A" myelocytes, 2 per cent; eosinophiles, 1 per cent; primitives, 1 per cent; plasma cells, 1 per cent. There were 7 nucleated red blood cells per 100 myeloid cells. The urine was negative. The Kahn reaction was negative.

(Continued on opposite page.)

restored to more normal levels; in every case this second marrow examination showed fewer myeloblasts and myelocytes than did the original.

Table 6 shows not only the millicuries of P^{32} required to restore the leucocyte count to approximately normal levels, but also the amount given each year or fraction of a year thereafter in an attempt to keep the blood in as normal a state as possible. Eleven patients have been followed for more than a year since the leucocyte count was originally restored to the range of 4,000 to 15,000 cells per cubic millimeter; in all but one of these patients the leucocyte count subsequently rose to above 20,000 cells per cubic millimeter and further therapy was therefore given. The millicuries of P^{32} administered during this first "post-remission" year varied between 1.5 and 18.8 millicuries. Four of these patients have been followed for two years following the initial hematologic remission and have required from 1.6 to 11.9 millicuries during the second year. Only two patients have been followed for three years since their initial remission.

Charts showing the hematologic response of two of our patients to P^{32} therapy are shown in Figs. 6 and 7. The first of these represents a fairly typical case of chronic myelogenous leukemia; the patient is still living and the disease has been under good control for two years. The dosage of P^{32} which this patient has received is also about average. The second patient was thought to be in an acute or terminal phase of his illness when treatment was begun. Although the remission induced was of brief duration, the chart is presented to emphasize that even in this stage of the disease therapy may be of value.

2. Effect of Therapy on the Clinical Manifestations of Myelogenous Leukemia: The effect of radioactive phosphorus on the symptoms of these thirty-nine patients with myelogenous leukemia is shown in Table 7. Every symptom was included in this table, even though many of them were probably caused by incidental disorders other than the leukemia. Weakness, by far the most common symptom, was completely or partially relieved in 89 per cent of the patients. All symptoms except coughing and nervousness were completely relieved in at least 50 per cent of the patients. Of the four patients who complained of coughing, one was a heavy user of tobacco, and the other three were in the terminal phase of the disease when P^{32} therapy was started. Three of the nine patients who complained of nervousness had had this symptom virtually all their lives.

In Table 8 is summarized the effect of therapy on the abnormal physical signs. Splenomegaly is the only abnormal physical sign which did not disappear completely in at least 50 per cent of patients. The spleen was originally palpable in thirty-six of these patients; it became no longer palpable in ten patients (27.5 per cent), and definitely decreased in size but remained palpable in twenty-three patients (64 per cent). In only one instance where the patient lived longer than

Course: The patient has had practically no symptoms since the onset of therapy with P^{32} . Within six weeks the spleen became no longer palpable; only on rare occasions since then has the tip of the spleen been felt on deep inspiration. She has had intermittent episodes of fatigue and nervousness, but her husband was in the Army, and she had four children to care for in addition to working regularly as a telephone switchboard operator. She is still alive and well (October, 1945).

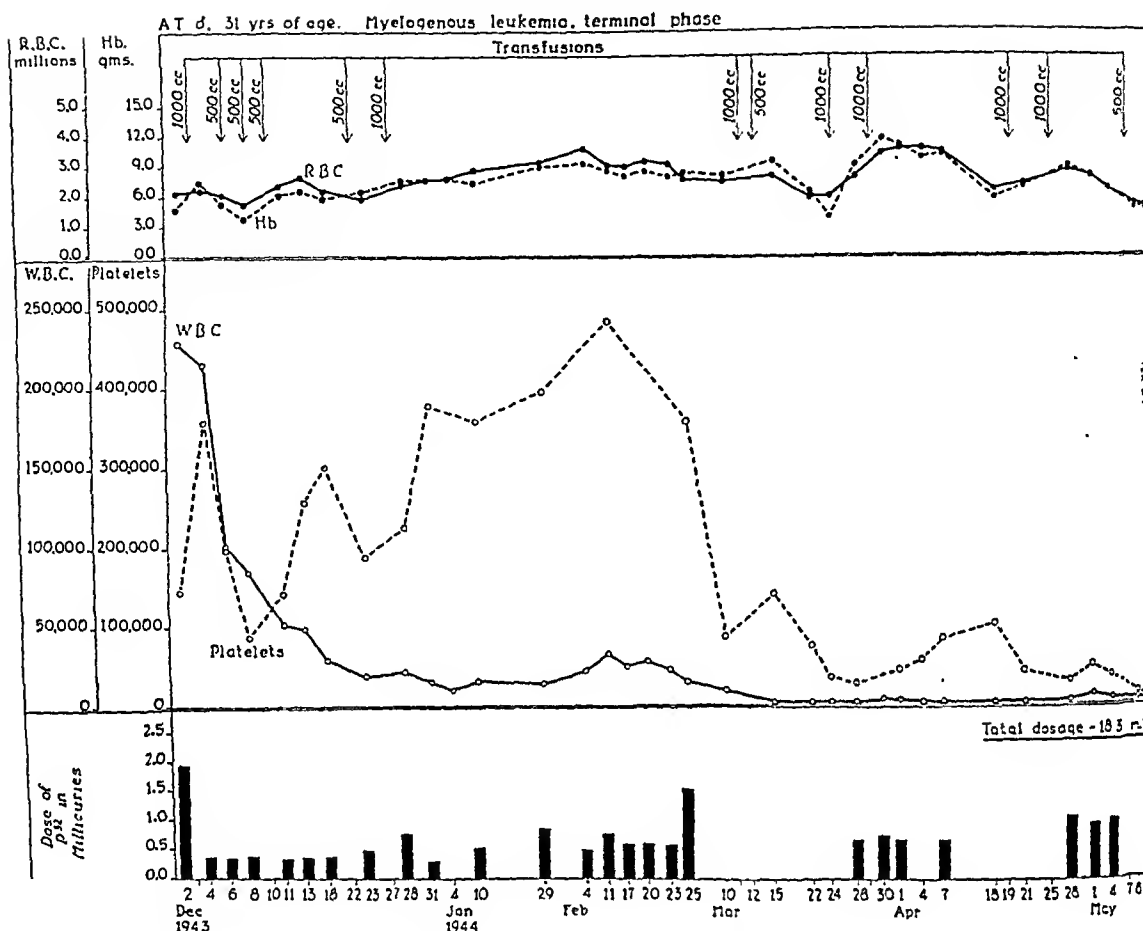


Fig. 7.—Terminal phase of myelogenous leukemia. Effect of P^{32} therapy on the blood.

Case Summary.—A. T., a man 31 years of age, began to have pain in both thighs and groins early in February, 1943. Shortly thereafter he had three shaking chills. The leucocyte count was found to be 266,000. Between February 23 and March 8 he was given x-ray therapy totaling 5,040 roentgen units. His symptoms improved and by June the white blood cell level had dropped to 5,800. He still had frequent headaches, cough, and persistent fever. In August, progressive anorexia occurred. The leucocyte level gradually rose, and, by September, 1943, reached 80,000. He was again given x-ray therapy, this time totaling 2,240 roentgen units. The white blood cell level decreased, but symptoms did not improve. During November, 1943, he developed progressive anorexia, severe pain in the chest, palpitation, dyspnea, orthopnea, increasing fever, and night sweats. Several days before he came to St. Louis for the first time he became mentally confused, and, at times, delirious. He was admitted to Barnes Hospital Dec. 2, 1943.

Physical Examination (Dec. 4, 1943): The temperature was 39.2° C. and the patient was semistuporous. There were several hemorrhages in the left fundus. The spleen measured 14 cm., and the liver 8 cm. below the left and right costal margins respectively (in mid-clavicular line). The lymph nodes were not enlarged. There was slight pitting edema of the feet.

(Continued on opposite page.)

TABLE 7. THIRTY-NINE PATIENTS WITH MYELOGENOUS LEUKEMIA—SYMPTOMATIC RESPONSE TO P³² THERAPY

SYMPTOM	NUMBER OF PATIENTS WHO HAD SYMPTOM AT ONSET OF THERAPY	% OF PATIENTS COMPLETELY RELIEVED OF SYMPTOM	% OF PATIENTS PARTIALLY RELIEVED OF SYMPTOM	% OF PATIENTS UNIMPROVED OR WORSE	% OF PATIENTS WHO LIVED LESS THAN 5 DAYS
Weakness and fatigability	36	53.5	33.5	5.5	5.5
Pounding in head	1	100	0	0	0
Dizziness	6	50	16.5	33.5	0
Dyspnea on exertion	16	50	25	18.5	6.5
Palpitation	8	62.5	25	12.5	0
Headaches	3	100	0	0	0
Tinnitus	1	100	0	0	0
Spontaneous bruising	4	50	25	25	0
Bleeding from nose or gums, or vagina (excessive)	9	89	0	11	0
Diarrhea	2	100	0	0	0
Bloating, dyspepsia	19	58	26.5	10.5	5
Nausea and/or vomiting	8	75	0	25	0
Anorexia	12	58.5	16.5	8.5	16.5
Abdominal pain	10	60	20	10	10
Cough	4	0	0	100	0
Blurring of vision	2	100	0	0	0
Pain in extremities	7	100	0	0	0
Soreness of gums	1	100	0	0	0
Semiconsciousness or stupor	1	100	0	0	0
Night sweats	1	100	0	0	0
Chills and feverishness	5	60	20	0	20
Nervousness	9	22	44.5	33.5	0
Swelling of legs or genitals	8	75	12.5	12.5	12.5

Laboratory Data: Blood counts (Dec. 2, 1943): leucocytes, 227,000; erythrocytes, 2,070,000; hemoglobin, 4.7 Gm.; reticulocytes, 2.0 per cent; platelets, 1,450,000; differential: segmented polymorphonuclear neutrophils, 34 per cent; stabs, 4 per cent; juveniles, 7 per cent; myelocytes, 36 per cent; myeloblasts, 13 per cent; monocytes, 1 per cent. Sternal bone marrow examination was not done on admission (patient too ill). Urine was negative except for a trace of albumin. Kahn reaction was negative.

Course: Sulfamerazine therapy was started December 4 and for the next month the blood level was maintained between 5 and 10 mg. per cent. During the first few days in the hospital he had repeated epistaxes. On the sixth day of therapy with P³² he began to feel stronger and from then on showed progressive symptomatic improvement. As the leucocytic level decreased, the differential improved, and when he was discharged from the hospital thirty-nine days after admission, there were only 3 per cent myelocytes and no myeloblasts in the blood. During the next two and one-half months he continued to feel quite well except for moderate fatigability. He continued to run a low-grade fever. On March 11 and 12 he had several blood transfusions accompanied by severe reactions; thereafter the temperature rose to 39° C. every afternoon. He became gradually weaker, lips and gums became sore, and he had frequent epistaxes. During April, 1944, he became jaundiced. The gums began to bleed and numerous petechiae appeared. He died five and one-half months after the initial P³² therapy.

At post-mortem examination there were numerous petechiae in the skin, mucous membranes, and serous surfaces; the spleen weighed 1,600 grams, the liver 2,800 grams, and there was generalized hyperplasia of the lymph nodes. There was a focal atelectasis and congestion of all lobes of the lungs. Sections of sternal, rib, vertebral, and femoral marrows showed a slight increase of the myeloid elements of the marrow; a few foci of erythropoiesis and a few megakaryocytes were seen.

TABLE 8. THIRTY-NINE PATIENTS WITH MYELOGENOUS LEUKEMIA—CHANGES IN PHYSICAL SIGNS FOLLOWING P³² THERAPY

SYMPTOM	NUMBER OF PATIENTS WHO HAD ABNORMAL PHYSICAL SIGN AT ONSET OF THERAPY	% OF PATIENTS IN WHOM ABNORMAL FINDING COMPLETELY DISAPPEARED AFTER THERAPY	% OF PATIENTS IN WHOM ABNORMAL FINDING PARTIALLY SUBSIDED	% OF PATIENTS IN WHOM THERE WAS NO IMPROVEMENT	% OF PATIENTS IN WHOM DEATH OCCURRED IN LESS THAN 5 DAYS
Fever	14	61.5	7	21.5	7
Tachycardia	19	58	21	16	5
Petechiae or ecchymoses	7	71	0	29	0
skin					
mucous membranes	3	100	0	0	0
Bleeding from nose or gums	2	100	0	0	0
Enlarged lymph nodes	26	50	23	23	4
Splenomegaly	36	27.5	64	3	5.5
Hepatomegaly	29	51.5	27.5	14	7
Systolic cardiac murmur	19	63	16	10.5	10.5
Edema of extremities	6	50	33	17	0
Ascites	2	100	0	0	0

five days after therapy was started did the spleen fail to decrease in size, at least temporarily. Twenty-six patients had palpably enlarged lymph nodes, but in twelve of these the enlargement was minimal (largest nodes, from 0.5 to 1 cm. in diameter). The lymphadenopathy disappeared entirely in thirteen patients (50 per cent of those having this abnormality), and the lymph nodes decreased in size in six (23 per cent).

The results are recorded for each patient in Appendix Tables 2 and 3. Only the initial response to therapy is tabulated. As the disease progressed, both symptoms and physical signs which had at first improved or disappeared tended to recur. For example, practically all of the patients who have died experienced extreme weakness just prior to death. In a few instances an enlarged spleen which decreased greatly during the early stages of treatment subsequently enlarged again until the organ was bigger than it had been at the time treatment was first started.

Two of the patients in this series had positive Kahn reactions which were almost certainly "false positives." One of these two was a housewife, 37 years of age, who had been married for nineteen years. She had no history of primary or secondary syphilitic lesions, and no signs or symptoms of cardiovascular or neurosyphilis. Blood obtained from her husband and son gave negative Kahn reactions. Her leukemia responded well to therapy, and following restoration of the blood cytology to approximately normal levels, her Kahn reaction became negative. The other patient was a minister, 76 years of age. Again there was no history nor any physical signs suggestive of syphilis. This patient responded very poorly to treatment. His total leucocyte count dropped to approximately normal levels, but the profound anemia persisted in spite of repeated blood transfusions. The Kahn test was repeated five months after treatment was started and was again positive.

One patient had tremendously enlarged lymph nodes, liver, and spleen which failed to decrease in size following P³² administration but diminished

markedly following a subsequent course of x-ray therapy. At the onset of treatment, this patient, a 26-year-old man, had no complaints except weakness, and progressive enlargement of nodes in the cervical, axillary, epitrochlear, and inguinal regions. Some of these nodes were from 2 to 3 cm. in diameter. The spleen measured 8 cm. and the liver 9 cm. below the left and right costal margins, respectively, in the mid-clavicular lines. The leucocyte count was 26,000 per cubic millimeter, the differential showed 12 per cent juveniles and 10 per cent myelocytes, the erythrocyte level was 5,170,000, and platelets 486,000 per cubic millimeter. He was given 5.94 millicuries of radioactive phosphorus during a period of thirty-four days. At the end of this time the leucocyte count was 7,800 per cubic millimeter with only 6 per cent juveniles and 1 per cent myelocytes, the erythrocyte level was down to 3,750,000, and the platelet level remained normal. In spite of this lowering of the white blood cell level, the axillary and femoral nodes had increased about 50 per cent in size, the spleen now measured 9 cm., and the liver 9.5 cm. below the costal margins. No further therapy was given during the next two weeks, and the axillary nodes enlarged slightly. The patient developed dyspnea and coughing; fluoroscopic examination revealed enlargement of upper mediastinal lymph nodes. X-ray therapy was then started; during the next three weeks the patient received 600 roentgen units (2 doses of 300 roentgen units) to each of the following ports: right neck, left neck, anterior mediastinum, posterior mediastinum, left upper quadrant, right upper quadrant, left groin, and right groin. During this course of treatment, the cervical lymph nodes decreased considerably in size, and the inguinal nodes decreased slightly. The liver and spleen did not diminish in size. Following x-ray treatment, the leucocyte level ranged between 1,900 and 4,400 cells per cubic millimeter, the erythrocyte count dropped to 1,195,000, and platelets virtually disappeared from the blood. The patient developed enormous anasarca and died twelve days after the last x-ray treatment. In Fig. 8 is illustrated the response of the enlarged cervical nodes to x-ray therapy after P^{32} had failed to produce any improvement. This reduction in the size of the cervical nodes relieved the choking sensation temporarily, but there was no other evidence that the therapy was beneficial. Undoubtedly, the dosage of radiation delivered to the involved nodes was the determining factor in this response. This case is presented to emphasize that a mass of lymph nodes which is causing severe discomfort by virtue of its size can be reduced more readily by x-ray than by P^{32} . Thus, roentgen radiation can be used to supplement radioactive phosphorus therapy when a localized effect is needed.

C. Comparison of Radioactive Phosphorus With Other Forms of Therapy Used in the Treatment of Myelogenous Leukemia.—From the results obtained in the treatment of myelogenous leukemia, it appears that radioactive phosphorus (1) has not succeeded in curing any patients with the disease, (2) has very little effect on the course of patients with acute or subacute myelogenous leukemia, (3) produces clinical and hematologic remissions roughly comparable to those induced by x-radiation and Fowler's solution in nearly all subjects with the chronic form of the disease. Since the ability of all known forms of therapy to produce a cure or to influence favorably the acute form of myeloid leukemia can

be dismissed from further consideration, comparison of relative therapeutic value can be limited to (1) the undesirable reactions produced by each form of therapy, (2) the completeness of remissions induced, and (3) their effect in prolonging life.

The oral or parenteral administration of radioactive phosphorus does not produce any discomfort in patients and does not cause radiation sickness. Patients who have previously been treated with x-ray and have had radiation



Fig. 8.—Response of adenopathy to x-ray therapy after P^{32} failed to produce any improvement. A, Lymph node enlargement in a patient with myelogenous leukemia following P^{32} therapy. B, Same patient following completion of a course of x-ray therapy.

illness are most appreciative of the fact that they can be treated without experiencing this reaction. The principal toxic effects to be avoided are those of leucopenia, thrombocytopenia, or aplastic anemia;¹⁹ these are reactions which may also be produced by overdosage with x-radiation or Fowler's solution. There are no toxic manifestations comparable to those frequently produced by Fowler's solution: gastrointestinal disturbances, exfoliative dermatitis, or hepatic damage. We believe that in the treatment of myelogenous leukemia, as in polycythemia vera, radioactive phosphorus is the most pleasant form of therapy for the patient.

Clinical remissions induced by radioactive phosphorus are roughly comparable to those obtained with other forms of therapy. Symptoms are often completely relieved and most of the abnormal physical signs disappear. Several patients (Table 6, Patients 2, 15, and 31) have had remissions of a year or more during which their blood showed practically no leukemic changes, their symptoms were minimal, and no therapy was given. While it is rare to obtain similar results with Fowler's solution unless a maintenance dose of the drug is regularly given, comparable remissions are not unusual after x-ray treatment and may even occur spontaneously. In an occasional person, relief of joint or deep bone pain, or reduction in the size of lymph nodes which may be causing pressure symptoms, may not be as complete with radioactive phosphorus as with x-ray.

More experience will be required before the conclusion may unequivocally be drawn that radioactive phosphorus does or does not prolong the life of patients with myelogenous leukemia. It is already obvious, however, that the course of the disease is not greatly lengthened. The average duration of the disease from onset of symptoms to start of therapy with P^{32} in the twenty-one patients of our series who already have died was 32.6 months; the average duration from onset of symptoms to death was 42.4 months or 3.64 years. The average length of life during which P^{32} was administered, therefore, was only ten months, but these figures are weighted against the effectiveness of the isotope because all patients are included and some lived for only three to ten days after the initial injection. Also, some had previously been treated with x-radiation and their sensitivity to irradiation may have been decreased. Similar figures for the eighteen subjects still living should also be given: duration of disease from initial symptoms to first treatment with P^{32} , 15.1 months; duration from initial symptoms to Sept. 1, 1945, 31.1 months. Three of these patients have now been treated for more than three years and are still feeling well. When these data are compared with those published in the literature, however, they are not particularly impressive. Wintrobe^{48a, b} found that the average duration of life following the onset of symptoms in 259 patients with chronic myelocytic leukemia reported by various authors was 3.28 years. Nathanson and Welch⁴⁹ found the median life expectancy for such patients to be about forty months. Minot, Buckman, and Isaacs^{50a} reported that the average duration of life after the first symptom in fifty-two nonirradiated patients with chronic myelogenous leukemia was 3.04 years and in sixty-eight irradiated patients, 3.5 years. Individuals with the disease not infrequently live for more than five years. It appears, therefore, that radioactive phosphorus as it is used now does not greatly prolong the life of patients with chronic myelogenous leukemia; if it is any more effective than x-ray in this regard, the difference is measurable in terms of a few months and not in years.

D. Summary and Recommended Method for Administering P^{32} to Patients With Chronic Myelogenous Leukemia.—Our results corroborate the experience of previous investigators that in chronic myelogenous leukemia radioactive phosphorus therapy is effective in producing hematologic remission. The improvement in the blood and bone marrow cytology is accompanied in most cases by

striking relief of symptoms and marked improvement in the abnormal physical signs. The treatment does not produce radiation sickness, or any other subjective disturbances. The data on our patients suggest that radiophosphorus therapy is comparable to x-radiation with respect to prolongation of life.

In view of the extreme variation in the dosage of radioactive phosphorus required to bring about a restoration of the blood cytology to normal, no fixed schedule of treatment can be employed. However, the following routine has been found satisfactory for the first two weeks of therapy:

DAY OF TREATMENT	DOSAGE IN MC. (INTRAVENOUS)
1	1-2
3	0.5-1
6	0.5-1
10	0.5-1
14	0.5-1

The weight of the patient, the extent of the leucocytosis, and the degree of bone marrow hyperplasia are the determining factors in deciding whether to employ the smaller or the larger dosage. After the second week, and at times even before then, treatment must be individualized, depending upon the initial response. In patients having a very high initial leucocyte level, a safe procedure is to continue to administer from 0.5 to 1.0 millieuries at weekly intervals until the white blood cell level drops to approximately 30,000 cells per cubic millimeters. Further administration is then either withheld, or the dosage is decreased and the interval between injections increased. If the radiophosphorus be given orally, the above-mentioned doses should be multiplied by $\frac{4}{3}$.

It is not implied that these recommendations represent the optimum treatment schedule. The ideal dosage per injection and the most satisfactory interval between injections are unknown. This tentative schedule merely represents one fairly satisfactory method of administering radiophosphorus. Actually, there was relatively little difference in the clinical or hematologic response of those patients who received small doses at frequent intervals and those who received larger doses at less frequent intervals. Furthermore, one usually can safely condense the course of treatment so that the leucocytes may be lowered to near-normal levels within two to three weeks rather than within the one to two months required with the more conservative schedule outlined. There is probably more danger of depressing erythropoiesis and platelet formation, however, when dosage is concentrated.

Several investigators have advocated that the amount of radioactive phosphorus to be given should always be calculated on the basis of millieuries per kilogram of body weight. In other words, an individual weighing 100 kilograms should receive exactly twice as great an initial dosage as would be given to a person weighing 50 kilograms. Although this is a theoretically sound conception, in actual practice body weight is of little value in determining the dosage for a given patient because of variation in susceptibility of leukemia cells to irradiation. In the last analysis, the optimum total dosage for any patient will depend on the observed response of that patient to therapy.

V. LYMPHATIC LEUKEMIA

The classification of the various types of lymphatic leukemia is a controversial subject. This is particularly true of that relatively large group of acute leukemias which many hematologists classify as leucosarcoma. Recognition of this particular type of acute leukemia is by no means universal. The distinction has to do with pathogenesis. If the disease is more closely related to lymphosarcoma than to chronic lymphatic leukemia, then its designation as leucosarcoma would seem to be preferable, whereas if the reverse is true, then acute lymphatic leukemia would seem more logical. As the etiology of all forms of leukemia is unknown, this controversy is of little practical importance. We have chosen, however, to use the terms leucosarcoma and acute lymphatic leukemia as defined by Wiseman⁵¹ in classifying our own patients. The distinction between leucosarcoma and subacute or acute forms of lymphatic leukemia is made primarily on the basis of the morphologic appearance of the cells in supravital stained preparations.⁵¹

In order to avoid confusion, both in discussing the literature and in analyzing our own cases, we shall consider all types of lymphatic leukemia under two headings: 1. *Chronic and Subacute Lymphatic Leukemia* and 2. *All Acute Types of Lymphatic Leukemia Including Leucosarcoma*.

A. *Review of the Literature.*—There are ten reports in the literature dealing with the therapeutic effectiveness of radioactive phosphorus for lymphatic leukemia.^{1-2, 5, 7, 8-10, 12, 15} They are based on the study of 120 patients of whom sixty-four had chronic or subacute lymphatic leukemia and fifty-six had some form of acute lymphatic leukemia (including leucosarcoma).

The first case was reported in Lawrence's original paper.¹ The patient was a girl, 24 years of age, who first noticed enlarged cervical lymph nodes in February, 1937. In May, 1937, the diagnosis of lymphatic leukemia was made, and she was given x-ray therapy. Further x-ray was given in August, and again in October of the same year. Following each course of therapy, the leucocyte level fell (to as low as 600 cells per cubic millimeter), only to rise again rapidly. Toward the end of November she developed ulcerations of the gums, a leukemic skin rash, fever, and pain in several joints. By Dec. 14, 1937, the white blood cell level had risen to 116,800 cells per cubic millimeter, of which 97 per cent were lymphocytes; the hemoglobin was about 50 per cent. Between Dec. 14, 1937, and Feb. 24, 1938, she was given orally seventeen doses of P₃₂ totaling 44.1 millicuries. The leucocyte level first decreased to 8,300 cells per cubic millimeter, but later rose to 93,000 in spite of continued treatment. Her general condition showed a progressive decline. Bleeding of gums and diarrhea occurred; she became comatose and died two days after taking the last dose of P₃₂.

Since this original report, specific data have been published for thirty-one cases of chronic or subacute lymphatic leukemia and for nineteen cases of the acute forms of the disease. Twenty-five of these chronic or subacute cases and sixteen of the acute ones were included in the comprehensive report of Erf, Tuttle, and Lawrence.⁵ A summary of the symptoms and physical signs before and after treatment was given for all of these patients, but hematologic data were summarized for only two of the acute and eight of the chronic cases. Of the twenty-five patients with chronic lymphatic leukemia, eight had partial and one had a complete remission; of the sixteen patients with acute lymphatic leukemia, only one had a partial remission and one had a complete remission. At the time of the report, thirteen of the patients with the chronic form of the disease and all but one of those with the acute form were dead. The authors concluded radiophosphorus is as effective in relieving the clinical manifestations as are other types of treatment in general use. In all eight of the patients with chronic

TABLE 9. SUMMARY OF REPORTS FROM THE LITERATURE ON PATIENTS WITH LYMPHATIC LEUKEMIA TREATED WITH P₃₂

AUTHOR AND REFERENCE	PATIENT	AGE AND SEX	APPROX. INITIAL W.B.C. COUNT (PER C.M.M.)	TREATMENT	COMMENTS
Warren ²	1	55, M	20,500	5 injections of P ₃₂ totalling 9.1 mc. during period of 5½ months	<i>Acute lymphatic leukemia.</i> When patient was first seen he had pneumonia, for which x-ray therapy was given to the left chest; following this, leucocyte level dropped from 118,500 to 20,500 on day P ₃₂ therapy was started; 13 days later W.B.C. count was 7,300; it remained normal throughout the 6 months he was followed; clinical condition remained "fair"
	2	68, M	42,550	5 injections of P ₃₂ totalling 13.1 mc. during period of 5 months	<i>Acute lymphatic leukemia.</i> Within 6 weeks leucocyte level dropped to 11,600; it remained at about this level throughout remaining 3½ months of observation; R.B.C. level rose but patient received 3 transfusions; when last seen, he was still working steady
	3	57, M	126,500	4.0 mc. given I.V. June 28; 1 mc. I.V. July 11	<i>Subacute lymphatic leukemia.</i> Total leucocyte count dropped to 77,750 on 11th day after treatment was started; differential continued to show 100% lymphocytes and bone marrow showed 96% lymphocytes
Kenney and associates ³	1	38, F (89% lymphoid cells in the bone marrow)	6,600	12.3 mc. given in 11 doses from 4/25/41 to 5/16/41	<i>Leucosarcoma.</i> Roentgen therapy given prior to P ₃₂ was ineffectual; P ₃₂ was likewise of no benefit, and patient died 1 month after this treatment was started
Kenney ³	1	Not given	20,000	16.1 mc. in 12 treatments during period of 120 days	<i>Chronic lymphatic leukemia.</i> Patient had just finished full course of x-ray therapy but without complete control of his disease; leucocyte level dropped to around 10,000, where it remained, and per cent of lymphocytes decreased from around 85 to about 65%
	2	58, M	27,200	9.1 mc. in 7 doses (from 11/19/40 to 1/20/41); 11.2 mc. in 6 doses (from 3/4/41 to 4/16/41)	<i>Chronic lymphatic leukemia.</i> Following P ₃₂ therapy there was virtually no change in either leucocyte count or per cent of lymphocytes; enlarged lymph nodes and spleen did regress in size, and symptomatically patient improved markedly
Low-Beer and associates ¹²	1	41, M	1,020,000	12.05 mc. in 19 doses (from 10/25/41 to 1/16/42); 6.48 mc. in 8 doses (from 3/18/42 to 8/15/42)	<i>Chronic lymphatic leukemia.</i> Within 1 month after start of P ₃₂ spleen and lymph nodes had markedly decreased in size, and there was pronounced clinical improvement; on 1/30/42 W.B.C. count was 9,900 and R.B.C. level had increased from 2,960,000 to 5,790,000; when last seen (Aug., 1942), patient was working regularly as watchman

TABLE 9—CONT'D

AUTHOR AND REFERENCE	PA- TIENT	AGE AND SEX	APPROX. INITIAL W.B.C. COUNT (PER C.M.M.)	TREATMENT	COMMENTS
Low-Beer and associates ¹²	2	60, F	92,400	Not given; stated only that radiation level was kept at 3,000 mc. for 2 months; 1 month later patient was given 2.1 mc., 2 months later, 0.48 mc.	<i>Chronic lymphatic leukemia.</i> Definite improvement in blood condition was observed within 12 days (counts not given); lymph nodes and spleen decreased in size; patient regained her original weight
Warren ¹⁵	1	49, M	296,000	Received 18.80 mc. orally (7 doses) and 17.98 mc. I.V. (8 doses) between 6/6/42 and 8/19/45	<i>Chronic lymphatic leukemia.</i> Lencocyte level dropped but remained elevated; patient had practically complete relief of symptoms and showed marked regression of enlarged lymph nodes, liver, and spleen. Patient was able to work steady

lymphatic leukemia for which hematologic data were given, the total leucocyte level showed a progressive lowering toward normal, but the percentage of lymphocytes decreased by more than 10 per cent in only five instances. The leucocyte level in one of the two patients with acute lymphatic leukemia for whom hematologic data were given rose from 3,000 cells per cubic millimeter before treatment to 6,000 per cubic millimeter three months later; in the other patient the white blood cells fell progressively from an initial count of 40,000 to 1,000 cells nine weeks after the first treatment. In both of these cases the percentage of lymphocytes decreased and the relative number of granulocytes increased following therapy. Erf and his associates concluded, as they had already done for myelogenous leukemia, that there was a less favorable response to therapy with P^{32} among those patients who had had previous x-radiation.

Detailed data have also been published by Warren,^{2, 15} Kenney and his associates,^{8, 9} and by Low-Beer, Lawrence, and Stone¹² for six other patients with chronic lymphatic leukemia and three with acute lymphatic leukemia. Brief summaries of all these cases are given in Table 9.

Craver⁷ treated eleven persons with chronic lymphatic leukemia and thirteen with acute lymphatic leukemia; no data were recorded for any of these patients. Most of the eleven patients with chronic lymphatic leukemia were well along in the course of their disease and had previously been given x-ray therapy. In only three, or possibly four, of the cases was there evidence that the disease was controlled by P^{32} therapy. The dosage for those patients who were treated for any considerable period of time varied from 0.15 to 0.35 millicuries per kilogram of body weight. Craver concluded that the favorable effects seen in chronic lymphatic leukemia were mainly the regression of enlarged lymph nodes and spleens; in general, the blood and bone marrow were not notably influenced. Of the thirteen patients with acute lymphatic leukemia, two were adults and eleven were children. No benefit from administration of P^{32} was observed in either of the adults or in any of the nine children who had died. Two children were still living at the time of the report. In one of these there had been a slight effect on the size of lymph nodes. The other child had been under treatment for four months and showed a remarkable remission. Details concerning the remission were not given, but at the end of the four-month period a relapse occurred.

Kenney⁹ treated eight children with acute or subacute lymphatic leukemia (the number in each category was not stated, and in making our analysis we put them all in the acute group as all were children and all but one died within four weeks). No data were given for any of these patients, but analysis of the results of treatment led Kenney to conclude that: "Radioactive phosphorus therapy has to date been no more effective in the acute or subacute leukemia of childhood than has any other form of radiation therapy; i.e., had no value at all."

Eight adults with chronic lymphatic leukemia were also treated with P^{32} by Kenney.⁹ A case history was given for one patient and hematologic data and therapy were presented in a chart for a second patient; both of these cases are summarized in Table 9. From his experience in the treatment of these eight patients, Kenney drew the following conclusions: (1) Radioactive phosphorus should be administered cautiously to patients with aplastic or infiltrated bone marrow; (2) it has not been effective in patients who have become radiation fast; (3) it reduced the enlarged lymph nodes in four patients and reduced the spleen in three patients; (4) the leucocyte count was decreased in one of eight patients; (5) the proportion of lymphocytes in the differential count was altered in one of eight patients; (6) symptomatically, five of the eight patients were improved; (7) there was no radiation sickness.

The effect of P^{32} on four patients with chronic lymphatic leukemia was analyzed by Fitz-Hugh and Hodes,¹⁰ but again no data were given. Two of the four patients with chronic lymphatic leukemia obtained good remissions, and one was still in excellent condition a year later. One very ill patient died shortly after the first administration, and the last patient had only recently been started on treatment at the time of the report. None of the four patients with acute lymphatic leukemia was benefited by radioactive phosphorus therapy.

The last report was that of Warren,¹¹ who studied twelve cases of chronic and subacute lymphatic leukemia and twelve cases of the acute form of the disease. The only one for which data were given is summarized in Table 9. Five of eight patients with chronic lymphatic leukemia, one of four with subacute lymphatic leukemia, and three of twelve with acute lymphatic leukemia were "helped." It is interesting that Warren reported a better response to therapy with P^{32} in those patients who had previously received roentgen therapy than in those who had received no x-ray; seven of eleven in the former group were "helped," whereas only two of thirteen in the latter group were benefited.

In summary, there is agreement among most of these investigators that P^{32} is effective in the majority of cases of chronic lymphatic leukemia in (1) restoring the leucocyte count to near normal levels, (2) reducing the size of enlarged lymph nodes and spleen, and (3) causing temporary amelioration of symptoms. Only three reports present sufficient data to permit comparison of the relative effectiveness of this form of therapy in chronic lymphatic leukemia and in myelogenous leukemia. Erf and associates⁵ observed five complete remissions among forty-six patients treated for chronic myelogenous leukemia, whereas only one complete remission occurred among twenty-five patients with chronic lymphatic leukemia. Furthermore, although a fall in the leucocyte level to near-normal values was fairly constant in both groups, a significant rise in the erythrocyte level occurred more frequently in the myeloid group. Craver,⁷ after treating eleven patients with each type of chronic leukemia, concluded that a favorable effect of P^{32} on the blood cytology was more constant in the chronic myeloid group. On the other hand, Warren¹¹ reported that 63 per cent of his patients with chronic lymphatic leukemia were helped by treatment, but only 47 per cent of the patients with chronic myelogenous leukemia were benefited.

B. Analysis of Results Obtained in Treating Forty-Five Patients With Lymphatic Leukemia.—Thirty patients with chronic or subacute lymphatic leukemia and fifteen patients with leucosarcoma (or acute lymphatic leukemia) have been treated with radioactive phosphorus. Of the patients in the former group, sixteen have died and fourteen are still living; all fifteen patients in the latter group have died.

1. Chronic and Subacute Lymphatic Leukemia: The same laboratory tests were done on these patients as on the subjects with chronic myelogenous leukemia. All persons with chronic lymphatic leukemia seen in this clinic in the last three and one-half years, except those who could not return for frequent follow-up visits, were treated with P^{32} and included in this series. The average duration of symptoms at the onset of therapy with P^{32} was nineteen months;

four of the patients had had specific symptoms referable to their leukemia for three years or longer. Thus, on the average, the duration of symptoms was shorter for this group of patients than for the group having myelogenous leukemia.

Hematologic and therapeutic data are summarized in Table 10. Twenty-one of the patients were men and nine were women. Only ten had had any other therapy prior to P^{32} ; in eight of these cases the previous treatment included roentgen radiation. In four instances, x-radiation had been administered within one to three months prior to the first injection of P^{32} .

The initial leucocyte count was above 15,000 cells per cubic millimeter in twenty-four patients, between 15,000 and 4,000 in five, and below 4,000 in one. Seventeen of the thirty patients had an initial anemia of less than 4,000,000 cells per cubic millimeter, and in fourteen instances platelets were less than 300,000 per cubic millimeter.

As with other blood dyscrasias, there was considerable variation in the dosage of radioactive phosphorus required to restore the total leucocyte count to approximately normal levels. Of the twenty-four patients whose total leucocyte level was originally above 15,000 cells per cubic millimeter, twenty showed a restoration to normal values. The total dosage required to restore the leucocyte count to approximately normal varied from 1.6 to 11.2 millicuries; fourteen of these twenty patients received less than 6 millicuries. Thus, the average dosage was somewhat smaller than in the group of persons with chronic myelogenous leukemia.

Twenty-four of the thirty patients showed a significant increase in the percentage of granular cells and a decrease in the percentage of lymphocytes in the blood following administration of P^{32} . Five who did not show this change had a poor clinical response to therapy; the sixth patient had a normal differential at the time P^{32} was first given (the diagnosis was based on the presence of a leucopenia and the characteristic bone marrow changes of chronic lymphatic leukemia).

The erythrocyte level increased by more than a million cells per cubic millimeter in only one of the twenty-three subjects who had received no transfusions. Six patients who were given transfusions showed a progressive fall in the erythrocyte level after treatment. The platelet count was less than 300,000 per cubic millimeter at the onset of P^{32} therapy in fourteen patients; in three instances the thrombocyte level rose above 400,000, but in no case did it rise as high as 500,000 per cubic millimeter after therapy. Thus, the improvement in the erythrocyte and platelet levels which occurred so frequently following treatment of myelogenous leukemia was rarely observed in individuals with lymphatic leukemia.

Ten patients have been followed for a year or longer since the leucocyte level was originally restored to normal. In no case was the remission sufficiently complete or prolonged so that the individual remained in remission for a year or more without further treatment. The dosage of P^{32} administered during this first "post-remission" year varied from 1.4 to 19.0 millicuries. Five of these

TABLE 10. HEMATOLOGIC DATA ON TWENTY-SIX

PATIENT	AGE AND SEX	PREVIOUS TREATMENT ^a	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED						P ³² THERAPY REQUIRED TO RESTORE W.B.C. COUNT TO APPROX. NORMAL (4,000-15,000)		MOST NORMAL BLOOD COUNTS AFTER P ³² THERAPY				
			W.B.C. (PER C.M.M.)	R.B.C. (MILLIONS PER C.M.M.)	HGB (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% GRANULOCYTES	% LYMPHOCYTES	MC.	DAYS	W.B.C. (PER C.M.M.)	R.B.C. (PER C.M.M.) ^b	HGB (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% GRANULOCYTES
1	32, M	X-R	98,500	4.41	12.8	620,000	10	89	11.2	34	8,900	4.17	12.5	401,000	72
2	63, F	X-R	63,500	4.88	15.4	600,000	13	86.5	4.06	54	8,100	4.46	13.0	775,000	20
3	35, M		71,800	5.17	16.3	899,000	12	85	6.55	36	7,550	4.88	15.0	504,000	60
4	67, F		4,450	2.05	6.6	90,000	39	61	3.02 ^d	134	5,100	2.97 T-5	9.7	174,000	52
5	58, F		16,700	4.15	11.7	880,000	55	40	2.926	15	10,000	3.97	11.0	670,000	63
6	58, M		40,300	4.06	9.4	382,000	18	81	4.931	33	11,050	4.56	10.7	467,000	53
7	58, M		132,000	2.00	6.7	172,000	9	89	6.912	28	8,050	3.64 T-2	12.1	210,000	33
8	51, M		18,100	4.72	14.1	370,000	60	35	3.987	29	8,300	4.53	13.6	795,000	67
9	31, M		21,050	4.00	13.5	632,000	41	57	3.433	28	17,350	4.06	11.0	755,000	70
10	46, M	X-R	17,500	5.10	15.8	816,000	47	48	1.642	33	11,700	5.08	15.8	920,000	59
11	60, M		471,000	1.83	4.6	91,000	2	98	9.654 ^c	68	24,200	2.67 T-8	7.8	150,000	3
12	70, M	X-R	12,150	3.84	12.7	415,000	30	63	7.023 ^d	112	5,900	4.16	13.7	458,000	54
13	75, M		444,000	2.82	10.5	705,000	3	96	9.012	112	14,500	4.05	11.5	1,027,000	51
14	53, M		28,500	4.11	12.7	788,000	15	81	2.718	30	6,400	4.25	14.4	1,207,000	51
15	66, M		13,650	3.45	10.5	569,000	20	80	2.367	7	8,550	3.77	11.9	360,000	64
16	62, M	X-R, T	6,750	0.95	1.0	260,000	15	82	8.446 ^d	36	8,400	1.35 T-2	3.8	153,000	18
17	47, M	X-R	11,300	4.25	12.9	391,000	31	63	7.412 ^d	533	7,650	4.58	13.4	484,000	52
18	59, F	Fo	81,500	2.45	7.2	201,000	7	91	5.010	14	7,850	3.24	10.7	480,000	42
19	39, M		23,900	4.40	13.4	290,000	46	49	5.667	10	10,550	4.00	12.1	385,000	45
20	64, M		59,700	4.04	13.5	218,000	9	90	10.852 ^c	127	18,350	4.23	13.0	370,000	11
21	48, F	X-R	37,000	3.70	12.9	244,000	5	95	8.87	159	10,500	4.26	13.1	--	19
22	53, F		310,000	3.30	10.9	152,000	0.5	99.5	9.364 ^c	48	325,000	2.68 T-5	8.8	32,000	1
23	47, M		2,700	2.25	6.3	90,000	52	34	2.361	6	5,100	3.65 T-11	11.7	476,000	66
24	54, M		36,500	4.61	15.0	837,000	10	88	6.799	49	5,600	5.56	14.9	555,000	68
25	56, M		66,500	1.08	3.8	119,000	6.5	92.5	4.156	12	4,200	3.33 T-11	8.9	138,000	46
26	52, F		382,000	3.07	9.6	708,000	1	98	18.865 ^c	107	354,000	3.25	9.2	868,000	1
27	55, M	X-R	150,000	2.80	9.2	232,000	4	96	4.131	31	8,550	3.48	10.1	306,000	72
28	59, F		55,500	3.97	11.6	553,000	14	85	3.570	35	8,450	4.15	12.0	521,000	34
29	55, F	T	195,000	1.62	5.8	140,000	3	97	5.135	22	8,100	3.11 T-5	11.3	480,000	52
30	50, M		142,000	2.04	5.8	32,000	2	98	4.231	22	4,400	2.08	5.8	64,000	24

^aX-R = X-ray therapy; Fo = Fowler's solution; B.M. = Bone marrow extract; T = Transfusions.^bT = Transfusions. The figure indicates the number of transfusions given.^cFigure represents the total number of millicuries administered, but leucocyte count never returned proximately normal (4,000 to 15,000).^dTotal leucocyte count approximately normal (4,000 to 15,000) at start of therapy. Figure represents total number of millicuries administered.

17TH CHRONIC LYMPHATIC LEUKEMIA

TREATMENT AND BLOOD COUNTS DURING FIRST YEAR OR FRACTION THEREOF FOLLOWING RESTORATION OF LEUCOCYTE COUNT TO APPROXIMATELY NORMAL (4,000-15,000)			TREATMENT AND BLOOD COUNTS DURING SECOND YEAR OR FRACTION THEREOF FOLLOWING INITIAL RESTORATION OF LEUCOCYTE COUNT TO APPROX. NORMAL			TREATMENT AND BLOOD COUNTS DURING THIRD YEAR FOLLOW- ING INITIAL RESTORA- TION OF LEUCOCYTE COUNT TO NORMAL			DURATION OF DISEASE (MO.) FROM FIRST SYMPTOM	
	W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)	M.C. OF P ₃₂	W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)	M.C. OF P ₃₂	W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)	TO START OF P ₃₂	TO DEATH OR 9/1/45
0	4,800-106,000	.72-3.94 T-9							36	49
3	13,400- 34,000	3.73-4.71	13.01	8,100-29,800	2.14-4.19	10.84	17,300- 33,400	3.66-4.46	14	53+
6	7,250- 27,700	3.95-5.00	8.38	6,000-25,700	3.75-4.70	10.90	800- 41,450	.90- 3.82 T-18	12	47
12	850- 5,100	.90-2.97 T-10							24	29
36	2,050- 10,050	2.22-3.64 T-1							8	11
77	11,050- 25,500	3.57-4.55	9.771	8,100-26,850	2.14-3.83	7.21	7,300- 30,150	2.22-3.27	3	37+
	7,750- 7	2.73-3.15 T-3							8	11
99	6,650- 43,350	2.95-4.53	10.60	4,300-67,000	2.38-4.37	1.358	3,650- 8,000	3.77-4.56	7	41+
	5,800- 7,700	2.99-4.06							18	20
33	11,450- 22,150	4.58-5.21	7.75	12,750-38,800	4.41-4.89				24	46
.65†	24,000-500,000	1.44-2.18 T-10							72	74
.026	4,100- 13,900	3.21-4.16	0	5,150-17,600	3.44-3.80	0	11,500- 17,000	3.01-3.42	12	35+
.17	9,400- 21,900	3.41-4.00							5	12
.18	3,500- 14,500	2.26-4.38							4	26+
.414	2,250- 8,550	2.17-3.77							18	41+
3.446†	4,850- 30,450	.58-1.35 T-13							5	6
7.368†	6,800- 35,500	4.16-4.79	0	5,100-16,600	4.03-4.45				21	42+
8.63	2,300-296,000	.99-3.24 T-16							15	22
1.712	10,500- 2,000	4.39-1.70 T-5							12	16
0.852	18,350- 51,000	3.02-4.23							12	16
4.31	3,300- 44,500	1.99-4.26 T-2							36	45
9.364	310,000-300,000	1.63-3.70 T-5							?	?
0.866	1,700- 4,800	.82-4.20 T-11							21	31+
0	3,900- 5,800	3.65-5.56							12-	21+
1.641	1,100- 5,500	1.06-3.33 T-19							1	5+
18.865	286,000-562,000	3.80-1.62 T-6							12	17
0	8,550- 14,400	2.86-3.13							120	123+
1.380	8,450- 13,400	3.62-3.94							1	5+
0	14,100- 4,300	2.61-1.50 T-6							8	11+
									18	19-

Duration from first treatment until death (or 9/1/45 if still living).

 Treatment in millieuries during the first twelve months (or fraction thereof) of P₃₂ therapy.

Patient is still living and working regularly two years after first treatment, but he has not been seen by

eight months after first treatment.

TABLE 10. HEMATOLOGIC DATA ON TWENTY

PATIENT	AGE AND SEX	PREVIOUS TREATMENT ^a	BLOOD COUNTS AT TIME P ₃₂ THERAPY WAS STARTED						P ₃₂ THERAPY REQUIRED TO RESTORE W.B.C. COUNT TO APPROX. NORMAL (4,000-15,000)		MOST NORMAL BLOOD COUNTS AFTER P ₃₂ THERAPY				
			W.B.C. (PER C.M.M.)	R.B.C. (MILLIONS PER C.M.M.)	HG (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% GRANULOCYTES	% LYMPHOCYTES	MC.	DAYS	W.B.C. (PER C.M.M.)	R.B.C. (PER C.M.M.) ^b	HG (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% GRANULOCYTES
1	32, M	X-R	98,500	4.41	12.8	620,000	10	89	11.2	34	8,900	4.17	12.5	401,000	12
2	63, F	X-R	63,500	4.88	15.4	600,000	13	86.5	4.06	54	8,100	4.46	13.0	775,000	12
3	35, M		71,800	5.17	16.3	899,000	12	85	6.55	36	7,550	4.88	15.0	504,000	6
4	67, F		4,450	2.05	6.6	90,000	39	61	3.02 ^d	134	5,100	2.97 T-5	9.7	174,000	5
5	58, F		16,700	4.15	11.7	880,000	55	40	2.92 ^c	15	10,000	3.97	11.0	670,000	6
6	58, M		40,300	4.06	9.4	382,000	18	81	4.931	33	11,050	4.56	10.7	467,000	28
7	58, M		132,000	2.00	6.7	172,000	9	89	6.912	28	8,050	3.64 T-2	12.1	210,000	3
8	51, M		18,100	4.72	14.1	370,000	60	35	3.987	29	8,300	4.53	13.6	795,000	6
9	31, M		21,050	4.00	13.5	632,000	41	57	3.433	28	17,350	4.06	11.0	755,000	7
10	46, M	X-R	17,500	5.10	15.8	816,000	47	48	1.642	33	11,700	5.08	15.8	920,000	1
11	60, M		471,000	1.83	4.6	91,000	2	98	9.654 ^c	68	24,200	2.67 T-8	7.8	150,000	4
12	70, M	X-R	12,150	3.84	12.7	415,000	30	63	7.023 ^d	112	5,900	4.16	13.7	458,000	1
13	75, M		444,000	2.82	10.5	705,000	3	96	9.012	112	14,500	4.05	11.5	1,027,000	4
14	53, M		28,500	4.11	12.7	788,000	15	81	2.718	30	6,400	4.25	14.4	1,207,000	1
15	66, M		13,650	3.45	10.5	569,000	20	80	2.367	7	8,550	3.77	11.9	360,000	1
16	62, M	X-R, T	6,750	0.95	1.0	260,000	15	82	8.446 ^d	36	8,400	1.35 T-2	3.8	153,000	1
17	47, M	X-R	11,300	4.25	12.9	391,000	31	63	7.412 ^d	533	7,650	4.58	13.4	484,000	1
18	59, F	Fo	81,500	2.45	7.2	201,000	7	91	5.010	14	7,850	3.24	10.7	480,000	1
19	39, M		23,900	4.40	13.4	290,000	46	49	5.667	10	10,550	4.00	12.1	385,000	1
20	64, M		59,700	4.04	13.5	218,000	9	90	10.852 ^c	127	18,350	4.23	13.0	370,000	1
21	48, F	X-R	37,000	3.70	12.9	244,000	5	95	8.87	159	10,500	4.26	13.1	--	1
22	53, F		310,000	3.30	10.9	552,000	0.5	99.5	9.364 ^c	48	325,000	2.68 T-5	8.8	32,000	1
23	47, M		2,700	2.25	6.3	90,000	52	34	2.361	6	5,100	3.65 T-11	11.7	476,000	1
24	54, M		36,500	4.61	15.0	837,000	10	88	6.799	49	5,600	5.56	14.9	558,000	1
25	56, M		66,500	1.08	3.8	119,000	6.5	92.5	4.156	12	4,200	3.33 T-11	8.9	138,000	1
26	52, F		382,000	3.07	9.6	708,000	1	98	18.865 ^c	107	354,000	3.25	9.2	868,000	1
27	55, M	X-R	150,000	2.80	9.2	232,000	4	96	4.131	31	8,550	3.48	10.1	306,000	1
28	59, F		55,500	3.97	11.6	553,000	14	85	3.570	35	8,450	4.15	12.0	521,000	1
29	55, F	T	195,000	1.62	5.8	140,000	3	97	5.135	22	8,100	3.11 T-5	11.3	480,000	1
30	50, M		142,000	2.04	5.8	32,000	2	98	4.231	22	4,400	2.08	5.8	64,000	1

*X-R = X-ray therapy; Fo = Fowler's solution; B.M. = Bone marrow extract; T = Transfusion
 *T = Transfusions. The figure indicates the number of transfusions given.

^cFigure represents the total number of millieuries administered, but leucocyte count never returned approximately normal (4,000 to 15,000).

^dTotal leucocyte count approximately normal (4,000 to 15,000) at start of therapy. Figure represents total number of millieuries administered.

CHRONIC LYMPHATIC LEUKEMIA

TREATMENT AND BLOOD COUNTS DURING FIRST YEAR OR FRACTION THEREOF FOLLOWING RESTORATION OF LEUCOCYTE COUNT TO APPROXIMATELY NORMAL (4,000-15,000)			TREATMENT AND BLOOD COUNTS DURING SECOND YEAR OR FRACTION THEREOF FOLLOWING INITIAL RESTORATION OF LEUCOCYTE COUNT TO APPROX. NORMAL			TREATMENT AND BLOOD COUNTS DURING THIRD YEAR FOLLOWING INITIAL RESTORATION OF LEUCOCYTE COUNT TO NORMAL			DURATION OF DISEASE (MO.) FROM FIRST SYMPTOM	
W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)		MG. OF P ₃₂	W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)	MG. OF P ₃₂	W.B.C. RANGE (PER C.M.M.)	R.B.C. RANGE ^b (PER C.M.M.)	TO START OF P ₃₂	TO DEATH OR 9/1/45
4,800-106,000 13,400- 34,000	.72-3.94 T-9 3.73-4.71		13.01	8,100-29,800	2.14-4.19	10.84	17,300-33,400	3.66-4.46	36 14	49 53+
7,250- 27,700	3.95-5.00		8.38	6,000-25,700	3.75-4.70	10.90	800-41,450	.90-3.82 T-18	12	47
850- 5,100 2,050- 10,050 11,050- 25,500	.90-2.97 T-10 2.22-3.64 T-1 3.57-4.55		9.771	8,100-26,850	2.14-3.83	7.21	7,300-30,150	2.22-3.27	24 8 3	29 11 37+
7,750- ? 6,650- 43,350	2.73-3.15 T-3 2.95-4.53		10.60	4,300-67,000	2.38-4.37	1.358	3,650-8,000	3.77-4.56	8 7	11 41+
5,800- 7,700 11,450- 22,150 24,000-500,000 4,100- 13,900	2.99-4.06 4.58-5.21 1.44-2.18 T-10 3.21-4.16		7.75 0	12,750-38,800 5,150-17,600	4.41-4.89 3.44-3.80	0	11,500-17,000	3.01-3.42	18 24 72 12	20 46 74 35+
9,400- 21,900 3,500- 14,500 2,250- 8,550	3.41-4.00 2.26-4.38 2.17-3.77								5 4 18	12 26+ 41+
4,850- 30,450 6,800- 35,500 2,300-296,000 10,500- 2,000 18,350- 51,000 3,300- 44,500 310,000-390,000 1,700- 4,800 3,900- 5,800 1,100- 5,500 286,000-562,000 8,550- 14,400 8,450- 13,400 14,100- 4,300	.58-1.35 T-13 4.16-4.79 .99-3.24 T-16 4.39-1.70 T-5 3.02-4.23 1.99-4.26 T-2 1.63-3.70 T-5 .82-4.20 T-11 3.65-5.56 1.06-3.33 T-19 3.80-1.62 T-6 2.86-3.13 3.62-3.94 2.61-1.50 T-6		0	5,100-16,600	4.03-4.45				5 21 15 12 12 36 ? 21 12+ 1 12 120 11 8 18	6 42+ 22 16 16+ 45 ? 31+ 21+ 5+ 17+ 123+ 5+ 11+ 19+

tion from first treatment until death (or 9/1/45 if still living).

nent in millicuries during the first twelve months (or fraction thereof) of P³² therapy.

it is still living and working regularly two years after first treatment, but he has not been seen by
ht months after first treatment.

patients have been followed for two years following the initial hematologic remission, and all but one of these received further therapy (8.4 to 13.0 millicuries) during the second year. Only one person has been under observation for a third year; she received 10.8 millicuries during this period.

The effect of P³² on the symptomatology of chronic lymphatic leukemia is shown in Table 11 (see Appendix Table 4 for complete tabulation). When first seen in our clinic, a good many of these patients had no complaints other than weakness and fatigability and enlargement of lymph nodes. Weakness and fatigability were completely or partially relieved in 78 per cent of individuals having these complaints; 22 per cent showed no significant improvement. Approximately one-half of all the symptoms encountered were completely relieved in at least 50 per cent of the patients. Not a single patient experienced

TABLE 11. THIRTY PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA—SYMPTOMATIC RESPONSE TO P³² THERAPY

SYMPTOM	NUMBER OF PATIENTS WHO HAD SYMPTOM AT ONSET OF THERAPY	% OF PATIENTS COMPLETELY RELIEVED OF SYMPTOM	% OF PATIENTS PARTIALLY RELIEVED OF SYMPTOM	% OF PATIENTS UNIMPROVED OR WORSE
Weakness and fatigability	27	41	37	22
Pounding in head	1	0	0	100
Dizziness	6	33	0	67
Dyspnea on exertion	9	44.5	44.5	11
Palpitation	3	67	0	33
Headaches	4	50	0	50
Spontaneous bruising	3	67	0	33
Bleeding from nose or gums or vagina (excessive)	5	40	0	60
Bloating, dyspepsia	5	0	40	60
Nausea and/or vomiting	1	0	0	100
Anorexia	9	0	55.5	44.5
Abdominal pain	3	67	0	33
Cough	3	67	33	0
Pain in extremities	4	75	0	25
Soreness of gums	2	100	0	0
Excessive sweating	5	40	0	60
Swelling of legs or genitals	1	100	0	0

TABLE 12. THIRTY PATIENTS WITH CHRONIC LYMPHATIC LEUKEMIA—CHANGES IN PHYSICAL SIGNS FOLLOWING P³² THERAPY

SYMPTOM	NUMBER OF PATIENTS WHO HAD ABNORMAL PHYSICAL SIGN AT ONSET OF THERAPY	% OF PATIENTS IN WHOM AB-NORMAL FINDING COMPLETELY DISAPPEARED AFTER THERAPY	% OF PATIENTS IN WHOM AB-NORMAL FINDING PARTIALLY SUBSIDED	% OF PATIENTS IN WHOM THERE WAS NO IMPROVEMENT
Fever	3	0	0	100
Tachycardia	7	0	14	86
Petechiae or skin ecchymoses	8	62.5	0	37.5
Enlarged lymph nodes	4	75	0	25
Splenomegaly	29	10	55	35
Hepatomegaly	24	21	58	21
Systolic cardiac murmur	16	37.5	25	37.5
Edema of extremities	10	0	20	80
Ascites	2	50	50	0
	1	0	0	100

complete relief of bloating, dyspepsia; or anorexia. In general, the symptomatic response to therapy was not as favorable as in the myelogenous leukemia group.

The effect of therapy on the abnormal physical signs is summarized in Table 12 (see Appendix Table 5 for complete tabulation). Hemorrhagic manifestations and edema of the extremities were the only two physical signs which disappeared entirely in as many as 50 per cent of the patients. An enlarged spleen became no longer palpable in only 21 per cent of the patients. Enlarged lymph nodes became no longer palpable in only 10 per cent, but in another 55 per cent there was some diminution in their size. In Fig. 9 are shown photographs of one of the patients before and after P^{32} therapy; the reduction in size of the cervical nodes following treatment is clearly visible. This patient was one of the 55 per cent in whom nodes diminished but never returned to normal size.



A.

B.

Fig. 9.—Patient 1, a 52-year-old man, with chronic lymphatic leukemia. *A*, Cervical lymph node enlargement three days prior to therapy. *B*, Same patient two months after therapy with P^{32} had been started. Patient had received 11.6 millicuries (all given within the first thirty-four days).

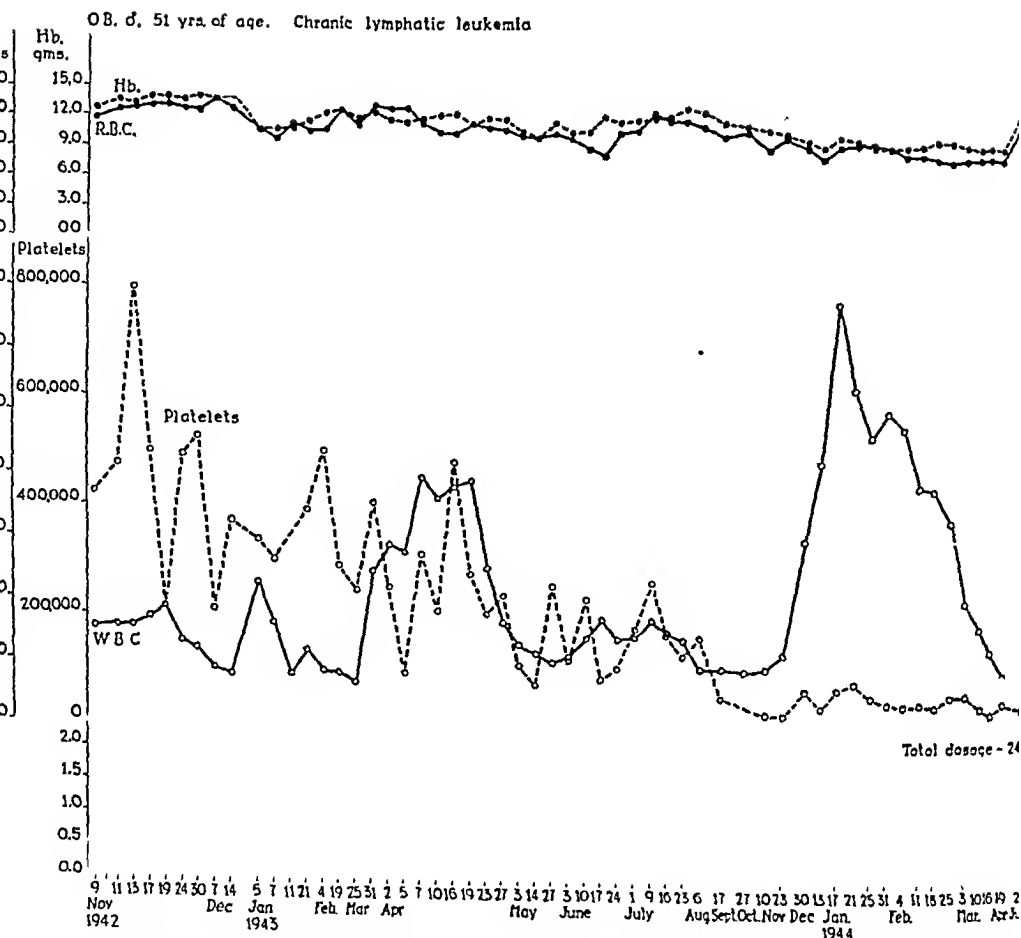


Fig. 10.—Chronic lymphatic leukemia. Hematologic changes following P₃₂ therapy.

Case Summary.—O. B., a man 51 years of age, first noted enlarged nodes in the neck and right axilla in April, 1942. In October, 1942, there was a rapid increase in the lymph node enlargement. He became short of breath and fatigued easily. His legs began to ache.

Physical Examination (Nov. 5, 1942): There was striking enlargement of the lymph nodes in the cervical, axillary, and inguinal regions; one mass in the right axilla measured 5 cm. in diameter and numerous cervical nodes measured from 3 to 4 cm. in diameter. There was a subconjunctival hemorrhage (right eye). The spleen measured 7 cm. and the liver 4 cm. below the left and right costal margins, respectively (mid-clavicular line).

Laboratory Data: Blood counts (Nov. 9, 1942): leucocytes, 15,200; erythrocytes, 3,910,000; hemoglobin, 12.6 Gm.; reticulocytes, 2.8 per cent; platelets, 422,000; differential: polymorphonuclear segmented neutrophils, 39 per cent; stabs, 8 per cent; eosinophiles, 2 per cent; basophiles, 1 per cent; monocytes, 6 per cent; lymphocytes, 44 per cent. Sternal bone marrow (Nov. 6, 1942): 91 per cent of the cells in the marrow were small lymphocytes, 3 per cent nucleated red blood cells. Urine, negative. Kahn reaction, negative.

Course: The patient felt progressively stronger and the lymph nodes decreased in size during the first six weeks of P₃₂ therapy. He then developed tracheobronchitis and pneumonitis and on Dec. 31, 1942, the leucocyte count was 31,700, with 71 per cent lymphocytes. Following sulfamerazine therapy and further P₃₂ he improved rapidly, and by the end of

(Continued on opposite page.)

In some patients the reduction in the size of the lymph nodes and spleen did not occur until many months after therapy with P^{32} was started. Patient 13 (Table 10) is a good example of this. Seven days after the onset of treatment, the lymph nodes showed little change in size; shortly after this they began to increase in size in spite of the fact that treatment was continued. On the fourteenth day of therapy (at which time the patient had received 4.1 millicuries of P^{32}), the lymph nodes in the neck and axilla were approximately 50 per cent larger than they had been initially. About a month later, following continued treatment, they began to recede, and two months later they were approximately one-half their original size.

In Patient 14 the cervical and axillary nodes were as large as 2 and 3 cm. in diameter, respectively. At first they decreased slightly in size, but later, even though administration of P^{32} was continued, the nodes again enlarged. Seven months after the beginning of therapy (total dosage, 10.9 millicuries), they were somewhat larger than they had been originally. At this time the leucocyte count had been normal for six months. The patient was then given, within a period of six days, 800 roentgen units of x-ray to each side of the neck and 400 roentgen units to each axilla. One month later the nodes had decreased about 50 per cent in size. It is now eighteen months since he was treated with the x-radiation. He has received no further therapy, is still working regularly, and is feeling fairly well. Unfortunately, we have not been able to persuade him to return to the clinic, and the present size of the nodes is unknown. Unquestionably, the lymph node enlargement responded more satisfactorily to x-radiation than to P^{32} .

Similarly, Patient 18 had tremendous splenomegaly which was totally unaffected by 13.1 millicuries of P^{32} given over a period of three months; the splenic measurement subsequently decreased from 25 cm. to 19 cm. below the costal margin following direct x-ray therapy to the spleen (two treatments of 400 roentgen units each). Bloating, belching, and anorexia remained prominent symptoms throughout the course of her illness.

The chief complaint of Patient 17 was soreness of the mouth and tongue. He had three firm, elevated, sharply demarcated plaques on the tongue; these

January, 1943, the blood cytology was within normal limits. He felt well until April, 1943, when the lymph nodes began to enlarge, and the spleen increased in size (measured 11 cm. below the costal margin). The leucocyte level rose to 43,300, with 78 per cent lymphocytes. At this time he felt very weak, had no appetite, and complained of pains in the legs and upper abdomen. Further P^{32} therapy was given, and again the symptoms subsided as the blood cytology returned to normal. He remained essentially asymptomatic until December, 1943, when he had "the flu." Weakness returned, gums became sore, lymph nodes enlarged, and numerous petechiae appeared on the trunk. By January 17, 1944, the leucocyte level was up to 67,000 (85 per cent lymphocytes) and the platelet count was 66,000. P^{32} therapy was intensified; the leucocyte returned to normal, but the platelet level dropped to between 50,000 and 15,000. He complained of pains in the back, legs, and left upper quadrant and had numerous petechiae and an occasional epistaxis. In April, 1944, he began to feel progressively stronger; all bleeding manifestations and other symptoms disappeared. He has remained essentially asymptomatic until the present (October, 1945). The lymph nodes on Oct. 31, 1945, were as large as 2 cm. in diameter; the spleen was felt just at the costal margin.

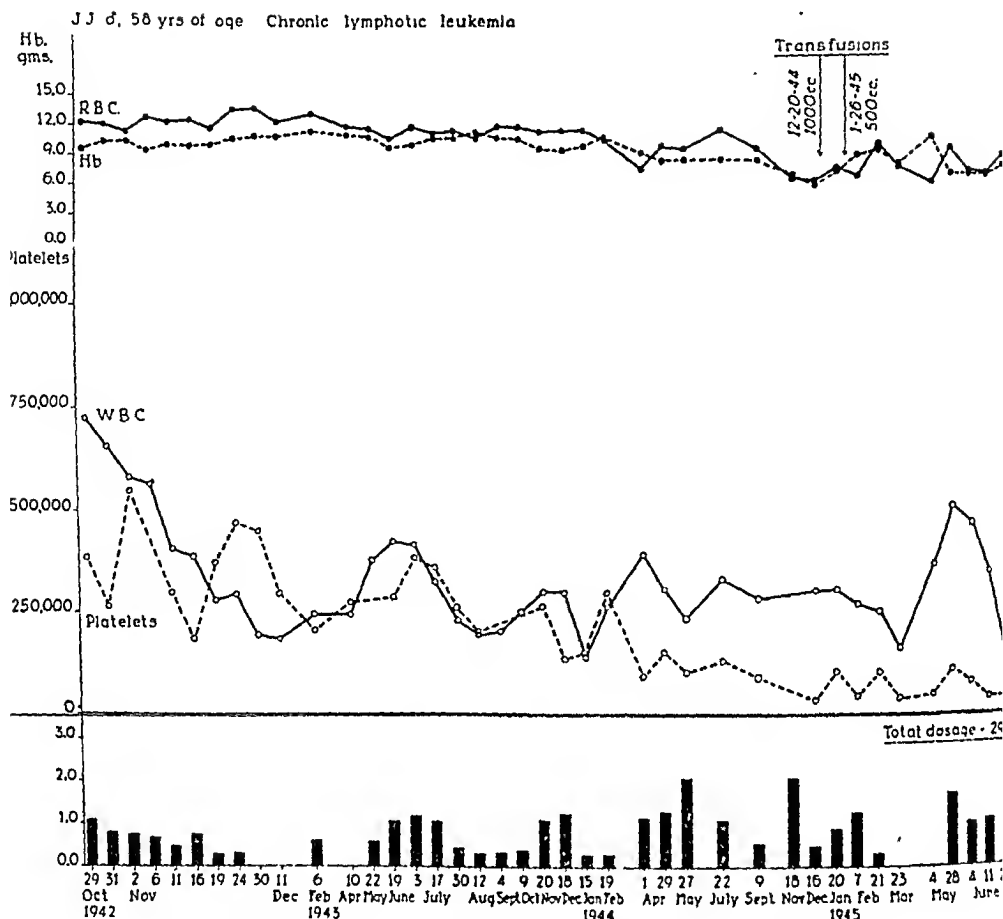


Fig. 11.—Chronic lymphatic leukemia. Response to P^{32} therapy.

Case Summary.—J. J., a man 58 years of age, developed weakness and fatigability in August, 1942, and these symptoms became progressively more severe. In October, 1942, he began to have aching pain in the left upper quadrant of the abdomen. Between June and November, 1942, he lost twelve pounds in weight. There were no other symptoms. He had been a coal miner for almost forty years.

Physical Examination: Patient's hair was white and he looked older than his stated age. There was moderate enlargement of the cervical, axillary, and inguinal lymph node (largest nodes, about 1.5 cm. in diameter). There were minimal changes of pulmonary emphysema. The spleen extended 8 cm. below the costal margin; the liver was not palpable.

Laboratory Data: Blood counts (Oct. 29, 1942): leucocytes, 40,300; erythrocytes, 4,060,000; hemoglobin, 9.4 Gm.; reticulocytes, 3.2 per cent; platelets, 382,000; differential mature lymphocytes, 79 per cent; immature lymphocytes, 2 per cent; segmented polymorph nuclear neutrophils, 11 per cent; stabs, 4 per cent; eosinophiles, 2 per cent; juveniles, 1 per cent; monocytes, 1 per cent. The sternal bone marrow (Nov. 2, 1942) was hyperplastic 74 per cent of the white cells were lymphocytes, and there were only 10 nucleated red cell per 100 white cells. Urinalysis was negative. The Kahn reaction was negative.

Course.—About one week after initial injection of P^{32} the patient began to feel stronger, and three weeks later he was almost entirely symptom-free. Two months after treatment was

(Continued on opposite page.)

were thought to have been caused by leukemia infiltration. He also had a severe infection of the gums with Vincent's organisms. The gingivitis healed, and the plaques entirely disappeared following P^{32} therapy. However, it is likely that extraction of all his teeth and local treatment with hydrogen peroxide mouth-washes and gentian violet applications were more important than the radioactive phosphorus in bringing about this improvement.

Two patients (Patients 12 and 13) had extensive skin eruptions diagnosed clinically and pathologically (skin biopsies) as leukemia cutis. In both instances the skin lesions improved markedly but did not disappear entirely following administration of P^{32} . Patient 12 has had several subsequent exacerbations followed by subsidence of the itching eruption.

Mention should be made of Patient 22, who was the outstanding example in this group of the complete failure of radiophosphorus therapy. This patient was a housewife, 53 years of age, who had had weakness and fatigability for seven years. For six months she had noted progressive weakness, progressive swelling of the abdomen with bloating, and marked anorexia. There were several bruises and petechiae scattered over the trunk; there was slight enlargement of the axillary and inguinal lymph nodes; the spleen measured 17.5 cm. below the costal margin and the liver 9 cm. (mid-clavicular line). The total leucocyte count was 310,000 cells per cubic millimeter; 99 per cent of these were lymphocytes. The erythrocyte level was 3,300,000; hemoglobin, 10.9 grams; reticulocytes, 8.0 per cent; and platelets, 152,000. The sternal bone marrow consisted of dense clumps of cells, 92 per cent of which were lymphocytes; practically no nucleated red blood cells were seen. The basal metabolic rate was +35 per cent.

She was given 6.7 millicuries of radiophosphorus over a period of thirty-one days, together with five blood transfusions. There was no improvement in any of her symptoms, and she soon began to bleed from the gums and vagina and into the retinae. The spleen increased slowly in size. The leucocyte level slowly increased, and at the time of death from a cerebral hemorrhage forty-nine days after treatment was started, the leucocyte count was 390,000 cells per cubic

started he was able to return to work in the coal mines (shoveling coal eight hours a day). At this time the lymph nodes were approximately half their former size, and the spleen now measured only 4 cm. below the costal margin. The patient had gained twelve pounds in weight.

He continued to be asymptomatic until July, 1943, when he developed pain in the right hip; at times this was accompanied by severe cramping pains in both thighs and calves. These pains cleared up spontaneously about two months later. Throughout the winter of 1943-1944 he felt quite well and continued to work regularly. In February, 1944, however, the lymph nodes and spleen began to enlarge; by April, 1944, the spleen measured 11 cm. below the costal margin. Toward the end of April he began to tire more easily, and numerous petechiae appeared. In July he became dizzy and began to have nose bleeds. These symptoms persisted, and in January, 1945, he had to quit his job. About this time he began to have severe pains in both shoulders and arms; an x-ray of the cervical spine revealed advanced osteoarthritis. From January to November, 1945, he continued to have intermittent fatigability, petechiae, nosebleeds, oozing from the gums, and occasional pains in the shoulders and hips. On November 17, 1945, the spleen measured 16 cm. below the costal margin.

TABLE 13. HEMATOLOGIC DATA ON FIFTEEN PATIENTS WHO

PATIENT	AGE AND SEX	PREVIOUS TREATMENTS	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED							TOTAL P ³² THERAPY ADMINISTERED (MC.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST INJECTION (DAYS)	BLOOD COUNTS AFTER CYTE COUNT		
			W.B.C. (PER C.M.M.)	R.B.C. (MILLIONS PER C.M.M.)	HG (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% LEUCOSARCOMA CELLS	% NORMAL LYMPHOCYTES	% POLYMORPHONUCLEAR NEUTROPHILES (INC. STABS)			W.B.C. (PER C.M.M.)	R.B.C. (MILLIONS PER C.M.M.)	HG (GM. PER 100 C.C.)
1	16, F	X-R	74,000	2.40	7.7	14,000	90	4	0	5,258	24	15,900	3.00	T-3 89
2	39, F	S	466,000	2.23	8.8	102,000	97	0	1	25,800	184	16,350	4.16	T-1 133
3	20, M	X-R, T	12,750	1.31	4.1	10,000	94	1	5	12,227	13	12,800	2.52	T-5 63
4	37, F	T	1,650	3.35	12.0	117,000	62	18	18	23,739	720	6,400	3.65	133
5	28, F	X-R, Fo	40,400	1.26	4.5	38,000	99	0	1	4,530	22	8,250	1.89	T-3 54
6	74, F	None	1,100	3.59	10.4	431,000	14	25	55	6,808	44	1,350	3.33	94
7	17, M	None	8,450	3.36	9.0	302,000	75	6	17	13,205	71	2,550	2.90	8
8	25, F	T	500	3.45	10.7	566,000	99	0	0	6,883	33	3,950	4.84	T-1 132
9	55, F	None	258,000	3.03	8.6	24,000	98	1	1	17,256	102	6,000	3.04	T-1 71
10	35, M	None	20,050	3.12	7.0	62,000	75	6	10	12,157	91	4,100	3.73	124
11	9, M	X-R	97,000	4.01	12.8	377,000	85	0	7	10,088	28	10,600	2.75	T-1 8
12	13, M	T	15,600	4.40	13.7	16,000	90	4	5	6,858	28	5,500	3.49	104
13	49, M	X-R	4,500	4.37	14.2	490,000	0 ^a	26	59	11,035	156	5,500	4.09	124
14	55, F	None	66,000	2.77	7.4	380,000	96	0	2	2,755	3	5,400	1.60	T-1 5
15	44, F	None	3,450	1.43	4.4	108,000	18	44	30	9,560	45	3,200	2.55	T-3 7

*X-R = X-ray therapy; S = sulfathiazole; T = transfusion; Fo = Fowler's solution.

^aIf the total leucocyte count became progressively more abnormal during the period of treatment the counts given in these columns represent the last counts prior to the terminal rapid rise (if leucocytosis) or fall (if leucopenia) of leucocyte count.

millimeter, of which 99 per cent were lymphocytes. Post-mortem examination revealed the usual lesions of lymphatic leukemia in the liver, in the spleen, and in all lymph nodes studied and extensive replacement of the bone marrow by lymphocytes. Only a few scattered foci of myelopoiesis and erythropoiesis were found in the marrow. The viscera were studded with petechiae, and there was extensive hemorrhage both into the ventricles of the brain and into the sub-arachnoid space. This case illustrates the fact that no benefit can be anticipated from P³² therapy in patients when the normal marrow tissues have been extensively and irreparably destroyed.

Two patients with chronic lymphatic leukemia who responded in a characteristic manner to therapy with P³² are illustrated in Figs. 10 and 11. In both of these patients a hematologic remission was obtained, and symptoms were ameliorated following treatment.

2. *Leucosarcoma or Acute Lymphatic Leukemia:* Of the fifteen patients with leucosarcoma included in this study, six were men and nine were women; all of them have died. The duration of symptoms prior to therapy with P³² varied from one to sixteen months (excluding Patient 13, in whom the duration was uncertain); the average duration was five months.

Five of the patients had had previous x-ray therapy. In Patient 3 (Table 13) this had produced no symptomatic improvement of any sort. In two other

LEUCOSARCOMA TREATED WITH RADIOACTIVE PHOSPHORUS

THERAPY (ON DAY TOTAL LEUCO AS MOST NEARLY NORMAL ^b)				BLOOD COUNTS IMMEDIATELY PRIOR TO DEATH							DURATION OF DISEASE FROM FIRST SYMPTOM		
PLATELETS (PER C.M.M.)	% LEUCOSARCOMA CELLS	% NORMAL LYMPHOCTES	% POLYMORPHONU- CLEAR NEUTROPHILS (INC. STARS)	W.B.C. (PER C.M.M.)	R.R.C. ^c (MILLIONS PER C.M.M.)	Hb (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% LEUCOSARCOMA CELLS	% NORMAL LYMPHOCTES	% POLYMORPHONU- CLEAR NEUTROPHILS (INC. STARS)	TO START OF P ₃₂ (WK.)	TO DEATH (WK.)	
21,000	71	23	4	15,900	2.21 T-5	6.5	2,000	98	1	0	16	20	
367,000	90	0	7	23,100	1.23 T-1	4.7	1,000	100	0	0	30	56	
32,000	96	1	1	550	1.10 T-5	3.6	4,000	50	30	10	14	161	
318,000	0	25	54	43,500	2.51 T-32	6.3	7,000	85	1	4	26	140	
19,000	81	1	10	350	1.44 T-6	3.9	6,000	90	0	10	64	68	
394,000	8	40	44	300	2.46 T-1	7.6	52,000	0	10	20	26	33	
162,000	0	58	42	750	2.82 T-5	10.2	25,000	76	0	16	24	34	
778,000	14	66	14	35,850	3.58 T-1	10.3	18,000	95	1	4	6	12	
24,000	43	18	5	3,650	.63 T-10	2.3	6,000	98	2	0	16	30	
498,000	6	16	68	80,000	2.36	7.5	38,000	95	0	4	12	24	
27,000	77	5	16	5,550	2.31 T-3	6.5	16,000	93	5	0	5	9	
210,000	86	3	7	350	1.67 T-4	5.0	21,000	44	44	0	29	33	
---	0	25	71	61,400	3.76 T-10	10.1	98,000	99	0	1	416 ^a	421	
4,000	76	12	10	5,400	1.60 T-1	5.6	4,000	76	12	10	4	51	
127,000	0	82	10	850	2.08 T-6	5.1	1,000	68	32	0	16	231	

*T = Transfusions. The figure indicates the number of transfusions given.

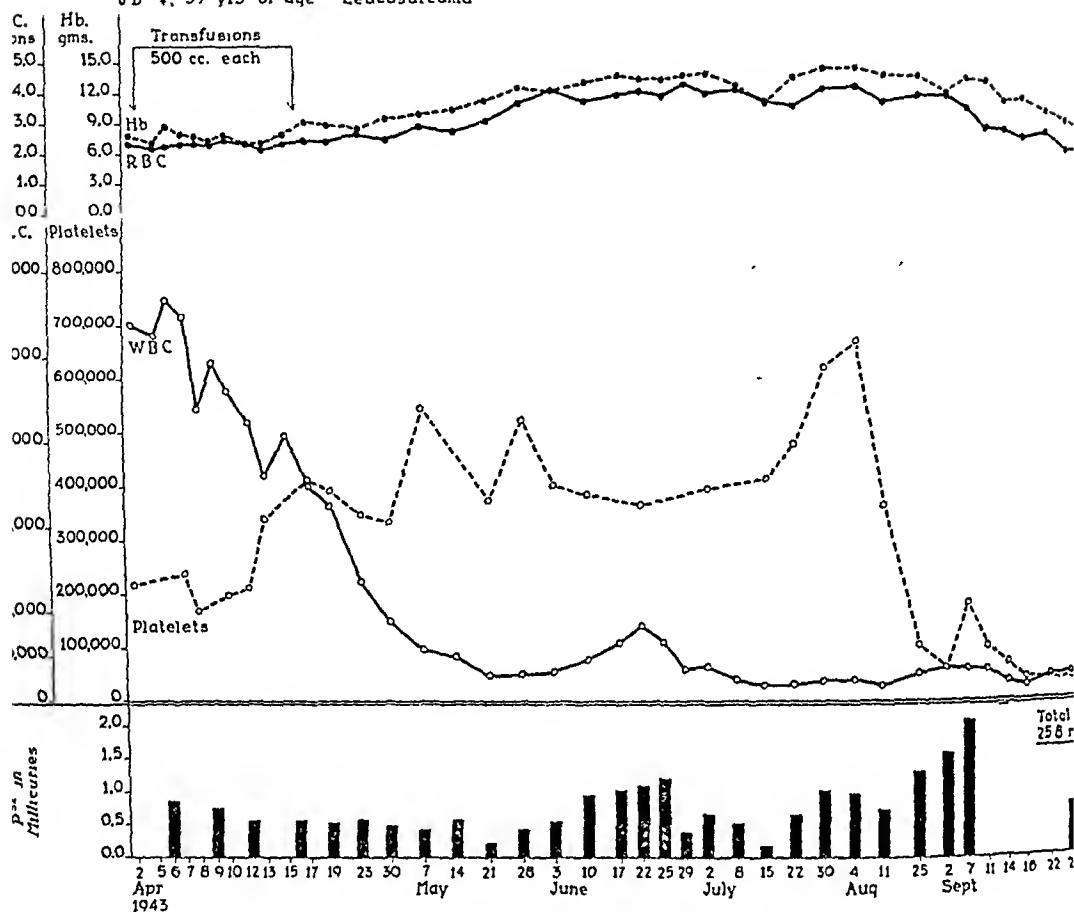
^bDiagnosis of leucosarcoma was made on the basis of bone marrow examination (50% leucosarcoma cells).

*Duration of lymphosarcoma.

instances, x-radiation had been followed by marked symptomatic improvement lasting one month (Patient 1) and five months (Patient 5). The fourth patient (Patient 11) was asymptomatic and was treated with x-radiation solely because he had developed enlarged nodes in the neck; following treatment these nodes decreased somewhat in size. The last of these patients (Patient 13) was a physician who had a remarkable history. Eight years prior to the time we first saw him he noted enlarged lymph nodes in the axillae and inguinal regions. A biopsy was performed in another city, and the diagnosis of lymphosarcoma was made.* X-ray therapy was given, and the lymph node enlargement completely disappeared. The patient remained subjectively well until four and one-half years later when he developed a lump in the left upper quadrant which was thought to be spleen. X-radiation was followed by complete disappearance of the mass. On both of these occasions repeated blood studies revealed no cytologic abnormalities. When we first saw the patient he still had perfectly normal leucocyte, erythrocyte, and differential counts; the diagnosis of leucosarcoma was established when 50 per cent of the cells in the sternal marrow were found to be leucosarcoma cells. Terminally, marked anemia, thrombocytopenia, and a moderate leucocytosis occurred; 99 per cent of the cells in the peripheral blood were leucosarcoma lymphocytes.

*The lymph node sections were studied by Dr. Robert Moore, Professor of Pathology, Washington University School of Medicine, who confirmed the diagnosis.

JB 9, 39 yrs of age Leucosarcoma

Fig. 12.—Leucosarcoma. Response to P^{32} therapy.

Case Summary.—J. B., a housewife, 39 years of age, first noted moderate fatigability in July, 1942; this progressed very slowly. In October, 1942, the ankles began to swell and she noted that she was becoming pale. In January she had bleeding of the gums, but this lasted only a few days. On March 9, 1943, she developed a severe sore throat with chills and fever. She was given sulfathiazole. Severe sore throat and high fever persisted. She became progressively weaker, and during the month of March she lost ten pounds. We first saw her on April 2, 1943.

Physical Examination: The patient was pale and listless. There were several large purpuric spots on the right thigh. The tonsils were large and fiery red, and there was a gray ulcerated area on the left tonsil. Small shotty nodes ranging up to 1 cm. in diameter were felt in the neck and both axillae. The spleen extended 18 cm. below the costal margin. A small amount of blood was seen coming from the cervical os.

Laboratory Data: Blood counts (April 2, 1943): leucocytes, 439,000; erythrocytes, 2,350,000; hemoglobin, 7.8 Gm.; reticulocytes, 1.0 per cent; platelets, 216,000; differential: 97 per cent typical leucosarcoma cells. The sternal bone marrow was hyperplastic, and 95 per cent of the white blood cells were leucosarcoma cells; 10 per cent of these were in mitosis. Fifteen normoblasts and 9 erythroblasts were seen per 100 myeloid cells. The urine was

(Continued on opposite page.)

TABLE 14. ANALYSIS OF SYMPTOMATIC RESPONSE TO P^{32} THERAPY OF FIVE PATIENTS WITH LEUCOSARCOMA

PATIENT	SYMPTOM BEFORE P^{32} THERAPY	MAXIMUM IMPROVEMENT IN SYMPTOMS	DURATION OF SYMPTOMATIC REMISSIONS	OTHER THERAPY
5	Profound weakness, severe soreness of gums, chills, anorexia	Anorexia disappeared entirely; all other symptoms improved	2 wk.	Blood transfusions
8	Severe sore throat, marked malaise, chills and feverishness, slight weakness	All symptoms completely relieved	3 wk.	One transfusion; severe pharyngitis and tonsillitis treated with penicillin
9	Marked weakness, profuse sweating, chills, moderate dyspnea and anorexia	All symptoms completely relieved except weakness	2 mo.	Transfusions; left lower lobe pneumonia was treated with sulfadiazine
12	Marked weakness and anorexia, severe pain in extremities, chills and feverishness	Pains in extremities completely cleared up and all other symptoms lessened in severity	3 wk.	None
15	Marked weakness, dyspnea, anorexia, severe pain in extremities, moderate cough and pain in chest	Moderate lessening in severity of all symptoms	1 wk.	Sulfadiazine, codeine, six transfusions

Various dosages of radioactive phosphorus were tried in the treatment of these patients. As noted in Table 13, the total dosage administered ranged from 2.7 to 25.8 millicuries; the P^{32} was usually given in relatively small amounts (0.5 to 1.0 millicuries) at fairly frequent intervals (once every two to five days). Since many of the patients had either normal leucocyte levels or a leucopenia when therapy was started, the white blood cell count was of little value as a guide to therapy. An attempt was made to regulate the dosage by serial bone marrow studies. Treatment was temporarily discontinued whenever a precipitous fall in the erythrocyte or thrombocyte levels occurred.

negative. Kahu reaction, negative. A swab from the throat revealed tremendous numbers of Vincent's organisms. On throat culture, the predominating organism was a pneumococcus.

Course: On April 4, 1943, sulfadiazine therapy was resumed (patient had been off the drug for three days); the sulfadiazine blood level was maintained between 5 and 10 mg. per cent for the next week, after which the dosage was decreased to 0.5 Gm. four times a day for another two weeks. P^{32} therapy was also started on April 4, 1943. The patient began to feel stronger about April 9, and the temperature gradually dropped until April 14, when it became normal. The spleen decreased in size, and on April 16 it measured 12 cm. below the costal margin. Her throat continued to be sore until April 25. Her strength gradually improved until about May 15, 1943, at which time she stated she felt perfectly well. She continued to be entirely asymptomatic until September 1. On June 10, 1943, the spleen extended 4.5 cm. below the costal margin. Early in September she again began to feel tired. About Sept. 9, 1943, she developed coryza, a cough, and pain under the sternum; she was again given sulfadiazine. These symptoms subsided, but early in October she developed increasing weakness, pain in the back, diarrhea, swollen tender gums, and fever. On Oct. 12, 1943, the diagnosis of right lower lobe pneumonia was made, and patient expired on Oct. 14, 1945. At post-mortem examination there was marked infiltration of all organs examined (except the pancreas) with leucosarcoma cells.

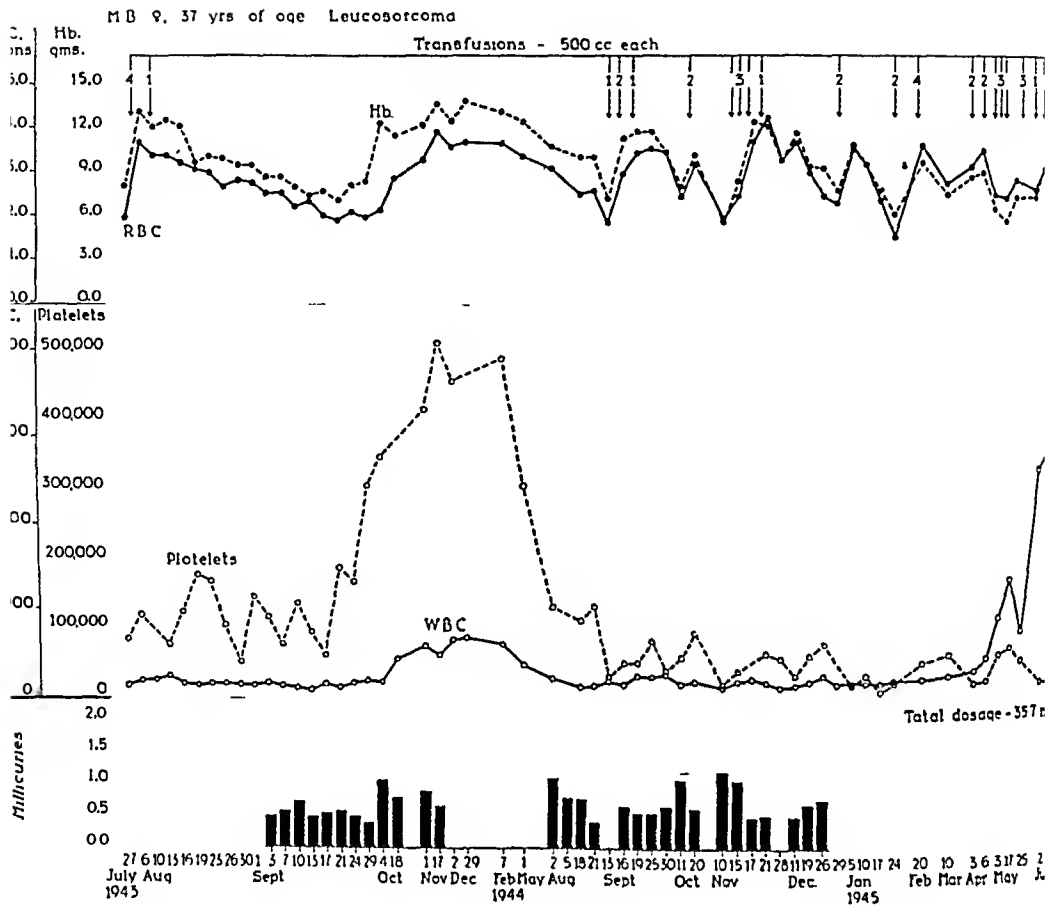


Fig. 13.—Leucosarcoma. Hematologic response to P₃₂ therapy.

Case Summary.—M. B., a housewife, 37 years of age, first noted moderate fatigability in August, 1942. This did not progress until February, 1943, when she became rapidly weaker and the ankles began to swell. She was hospitalized on March 7, 1943. Physical examination was negative except for pallor and a low-grade fever. The total leucocyte count was 1,000 cells per cubic millimeter; erythrocytes, 1,600,000; and platelets, 100,000. The bone marrow was found to be hyperplastic, and 94 per cent of the cells in the marrow were leucosarcoma cells. She was given eleven blood transfusions and was discharged May 23 without any other therapy. She was readmitted July 26, 1943, because she had developed a large perirectal abscess. She was too weak even to sit up in bed.

Physical Examination: The skin was strikingly pale. There was a large, red, hot, exquisitely tender area just to the left of the anus. There were no petechiae or ecchymoses, and the lymph nodes, liver, and spleen were not palpable.

Laboratory Data: Blood (August 10, 1943): leucocytes, 1,600; erythrocytes, 3,350,000; hemoglobin, 12.0 Gm.; reticulocytes, 1.8 per cent; platelets, 116,000; differential: polymorphonuclear neutrophils, 10 per cent; stabs, 8 per cent; monocytes, 2 per cent; adult, small lymphocytes, 18 per cent; leucosarcoma cells, 62 per cent. A second sternal bone marrow examination revealed 87 per cent leucosarcoma cells.

Course: The rectal abscess was incised and sulfamerazine therapy was started on Aug. 6, 1943. She received six blood transfusions. On August 10, therapy with radioactive

(Continued on opposite page.)

The cytologic changes in the blood following radiophosphorus therapy are summarized in Table 13. The leucocyte level dropped in all patients except two who initially had marked leucopenia. In the latter two instances, the leucocyte level increased progressively (to normal in Patient 4 and to 35,000 cells per cubic millimeters in Patient 8). All of these patients except one (Patient 10) received blood transfusions, in spite of which the erythrocyte level slowly fell in all but two. Nine of the subjects had an initial thrombocyte level of less than 300,000 per cubic millimeter; in all fifteen patients the platelet count was below 100,000 per cubic millimeter just prior to death, and in every instance but one the level was considerably lower than the pretherapy figure.

Eight patients in this group showed no significant improvement in any of their symptoms following therapy with P^{32} , and in most of them there was a rapid progression of all symptoms. In seven patients there was definite symptomatic improvement; four of these individuals became almost entirely symptom-free. However, in all but two the remission was of brief duration and could well have been due to transfusions, chemotherapy, bed rest in the hospital, or psychologic factors. The symptomatic responses of the five patients who had very brief improvement are analyzed in Table 14; the two patients who had more prolonged symptomatic remissions are not included in this table, as data on these individuals are given in detail in Figs. 12 and 13. Even in these

phosphorus was instituted. One month later, at a time when the patient had received 5.78 millicuries of radioactive phosphorus, the total white count was 659; only 20 per cent of these were leucosarcoma cells, whereas prior to treatment 62 per cent of the cells had been leucosarcoma cells. In spite of the profound leucopenia, the patient felt considerably stronger and the fever began to subside. During the next two weeks clinical improvement was progressive, weakness disappeared, and appetite became tremendous. She was discharged from the hospital seven weeks after treatment was started, at which time the total leucocyte count was just beginning to increase. Twelve weeks after treatment was started the total leucocyte count was 5,500 and less than 1 per cent of the cells in the peripheral blood were abnormal lymphocytes which we classified as leucosarcoma cells. A third bone marrow examination was done on the forty-sixth day of treatment, at a time when the total leucocyte count was only 1,200. The marrow showed a striking increase in myeloid and erythroid cells, and only 20 per cent of the cells were of the lymphoid series. Treatment was continued for three and one-half months; 11.9 millicuries of radioactive phosphorus were given. By this time the total leucocyte count, the differential, and the platelet count were all perfectly normal, and the red blood cell count had come up to 3,900,000 per cubic millimeter, with 13.6 Gm. of hemoglobin. The patient received no further therapy and the blood counts remained within normal limits for a period of five and one-half months. The white count then began to fall progressively and her symptoms returned; a second course of treatment was begun in August, 1944. She again improved symptomatically but the leucocyte count did not rise. She continued to have periods of relative freedom from symptoms alternating with periods of weakness, anorexia, and bleeding from the gums. She received occasional blood transfusions as indicated in the chart. In April, 1945, concomitant with a rise in the leucocyte count, she became weaker, began to bleed from the gums, and developed diarrhea, cramping abdominal pain, fever, and an abscess on her buttock. On May 17, 1945, sulfadiazine therapy was started. In June, 1945, extensive ecchymoses appeared over her body. The symptoms fluctuated in severity until July, 1945, when she became rapidly worse with increasing fever, anorexia, nausea and vomiting, and pain in the chest. She was found to have bilateral bronchopneumonia. On July 25, 1945, the spleen was palpable for the first time. She expired Aug. 9, 1945, exactly two years after treatment with P^{32} was started.

latter two instances where prolonged symptomatic and hematologic improvement occurred, other therapeutic measures were employed which certainly accounted for some of the improvement noted.

The changes which occurred in the physical signs following administration of P^{32} can be summarized very briefly. Of the fifteen patients, seven had ecchymoses or petechiae in the skin when first seen; in two of these individuals, this manifestation became much less marked and in the other five there was no improvement. Two individuals showed hemorrhages in the mucous membranes and retinae; these disappeared in one and became steadily worse in the other. Eleven patients had enlarged lymph nodes; these became no longer palpable in one patient and decreased in size in the other ten. Eleven subjects had an initial splenomegaly; the spleen became no longer palpable in one, decreased in four, and increased progressively in the other six. Hepatomegaly, originally present in six patients, showed slight improvement in three and no diminution in the other three.

C. Comparison of Radioactive Phosphorus With Other Forms of Therapy Used in the Treatment of Lymphatic Leukemia.—The therapeutic effectiveness of P^{32} should be compared principally with x-radiation because Fowler's solution is a relatively inferior therapeutic agent for the treatment of chronic lymphatic leukemia. The hematologic remissions produced by administration of P^{32} are incomplete in that the percentage of lymphocytes does not regularly return to normal even though the total white blood cell count frequently does. A similar statement may be made for x-ray therapy. Radioactive phosphorus is no more effective than x-radiation in relieving symptoms of patients with chronic lymphatic leukemia and often does not produce as much decrease in the size of lymph nodes and spleen as does x-ray. In none of our patients did P^{32} induce so complete a remission that further injections could be withheld for as long as a year. The remissions, therefore, were no better and may not have been as prolonged as is usual with x-radiation. Administration of radioactive phosphorus has one advantage in that it does not cause radiation sickness.

There was no indication that P^{32} is likely to prolong life more than does x-ray. The duration of symptoms to death or to Sept. 1, 1945, for the twenty-three patients who have been followed for a year or more (Patients 1 to 21, 23, and 24) was 31.8 months; nine of these persons are still living. They have been treated with P^{32} for an average of 14.4 months. The nine living patients in this group had had symptoms for an average of 12.4 months at the time P^{32} was first given and had an average duration of their disease on Sept. 1, 1945, of 36.3 months. When these figures are compared with the average duration of 3.29 years found for 152 cases of chronic lymphatic leukemia,⁴⁸ it is clear that the final figures for the duration of the disease in patients treated with P^{32} are not likely to exceed them by a significant margin. Minot and his associates⁵⁰ reported that the course of the disease was not appreciably lengthened by x-ray therapy.

Results in the treatment of acute lymphatic leukemia (including leucosarcoma) have been as disappointing as with all forms of therapy. An occa-

sional patient does have a relatively long, incomplete remission, but these may occur spontaneously and cannot yet be attributed to the therapeutic effectiveness of P^{32} .

D. Summary and Recommended Method for Administering P^{32} to Patients With Lymphatic Leukemia.—In our experience, somewhat smaller doses of radioactive phosphorus suffice to restore the total leucocyte level to normal in chronic lymphatic leukemia than in chronic myelogenous leukemia. However, the percentage of lymphocytes dropped below 50 per cent, and the percentage of granulocytes rose above 50 per cent in only sixteen of the thirty patients. Furthermore, a rise in the erythrocyte and platelet levels following P^{32} therapy was less frequent and much less marked among the patients with chronic lymphatic leukemia than among those treated for myelogenous leukemia. In addition to the less favorable hematologic response, symptoms and physical signs also responded less favorably in the individuals with lymphatic leukemia.

The chief advantage of radioactive phosphorus therapy over x-radiation in the treatment of chronic lymphatic leukemia is freedom from radiation sickness. It is our impression that therapy with P^{32} is probably as satisfactory as, but no better than, x-radiation in relieving symptoms and in prolonging life.

In general, the acute varieties of lymphatic leukemia are not significantly benefited by radioactive phosphorus. The few remissions observed could well have been due to blood transfusions, chemotherapy of infections, or could even have been spontaneous remissions.

The same schedule of therapy recommended for myelogenous leukemia can be employed for lymphatic leukemia except that, as a rule, administration should be discontinued earlier because of the more rapid fall in the leucocyte level in patients with lymphatic leukemia.

Seven of our patients with chronic lymphatic leukemia had an initial white blood cell level below 18,000 cells per cubic millimeter. Regulation of therapy for these individuals was more difficult. The severity of the disease in such individuals must be determined by the bone marrow cytology and the degree of associated anemia and thrombocytopenia. It is our feeling that roughly comparable dosages of P^{32} should be employed in the management of leucopenic leukemia as would be administered to patients with elevated leucocyte levels. An intelligent decision as to when therapy should be discontinued can be reached only by frequent determinations of the blood erythrocyte, platelet, and segmented polymorphonuclear levels and by serial bone marrow studies.

VI. MONOCYTIC LEUKEMIA

A. Review of the Literature.—The response of six patients with monocytic leukemia to therapy with P^{32} has been reported.^{1, 5, 15} Specific data are given for only three of these patients.

Lawrence and his associates¹ treated a 24-year-old woman who complained of progressive weakness of four years' duration, swelling of the lymph nodes of the neck, and furuncles on the forehead of three months' duration. Significant physical findings included pallor, generalized lymph node enlargement, herpetic lesions on the lips, bleeding of the gums, and a smooth liver edge palpable at the costal margin. When she was admitted to the hospital (Dec. 24, 1937), the total erythrocyte count was 2,400,000; hemoglobin, 60 per cent; leucocytes, 11,950, with 21 per cent polymorphonuclear cells, 22 per cent lymphocytes, and 50 per cent monocytes.

Treatment with radioactive phosphorus was started Jan. 13, 1938. By that time the white blood cell level had increased to 58,000. Between January 13 and February 18, the patient was given orally nine doses of radiophosphorus totaling 23.2 millicuries. The furuncles were incised and drained. She was also given iron by mouth and four blood transfusions. The leucocyte level rose during the first week of therapy to about 178,000 cells per cubic millimeter and then fell rapidly to approximately 15,000 cells, most of which were monocytes. New furuncles appeared, and stomatitis with ulceration and hypertrophy of the gums became progressively more severe. The spleen became easily palpable. Terminally there was a rise in temperature, and the white blood cell level rose to 71,000; 91 per cent of the white cells were monocytes. Death occurred forty-two days after therapy had been started.

Post-mortem examination revealed prominent infiltration of the liver, heart, colon, gall bladder, kidneys, uterus, and ovaries with monocytic cells. The lymph nodes, spleen, and bone marrow were composed largely of monocytes.

Erf and his associates⁵ tabulated data on a man, 40 years of age, who had had fatigue, anorexia, and cough for five months and abdominal pain for three weeks. The spleen was felt 1 centimeter below the costal margin and there were hemorrhages in the fundi. The total leucocyte count was 41,000 cells per cubic millimeter. Sternal marrow was hyperplastic and contained "many monoblasts." The patient was given 6.9 millicuries of radiophosphorus by mouth within a period of three days. Death occurred six days after the first dose. Post-mortem examination revealed reticulo-endothelial hyperplasia of most organs, including the kidneys and the submucosa of the gastrointestinal tract.

Warren¹⁵ treated four patients with monocytic leukemia, one of whom was "helped," but the other three showed no response. A case report was given for only one patient, a man, 31 years of age, who had had weakness, anorexia, and dyspnea for two months. The total leucocyte count was 6,500 per cubic millimeter; 50 per cent of the white cells were monoblasts. The patient was given 6.27 millicuries of P^{32} intravenously during a period of three weeks. Death occurred four weeks after the initial injection. Post-mortem examination was interesting in that the bone marrow was hypoplastic; most of the other organs were infiltrated with monocytes.

B. Analysis of the Results Obtained in Treating Ten Patients With Monocytic Leukemia.—Of the ten patients with monocytic leukemia whom we have treated with radioactive phosphorus, only one is still living. All of the patients were acutely ill when treatment was started, and eight of them remained in the hospital until they died. Data on these patients are summarized in Table 15. All had had rapidly progressive symptoms including painful bleeding gums, sore throat, fever, anorexia, sore, swollen cervical lymph nodes, and progressive prostration for from ten days to four weeks before admission to the hospital. Patients 3, 5, 7, and 8 felt well until the onset of this period of rapidly progressive symptoms; all of the other patients had noted weakness and fatigability without other manifestations for variable periods ranging up to six months prior to the onset of the acute phase. The average total duration of symptoms was eleven weeks. None of these individuals had been given any previous therapy except for four who had received transfusions. In seven instances transfusions were given during the period of radioactive phosphorus therapy.

The total dosage of P^{32} administered varied from 0.14 millicuries to 11.06 millicuries, with an average of 5.3 millicuries. In every instance except one (Patient 10), treatment was continued until the time of death.

The effect of therapy on the total leucocyte count was variable. The total white blood cell level increased in one-half the cases and decreased in the other

TABLE 15. HEMATOLOGIC DATA ON TEN PATIENTS WITH MONOCYTIC LEUKEMIA TREATED WITH RADIOACTIVE PHOSPHORUS

PATIENT	AGE AND SEX	PREVIOUS TREATMENT ^a	TOTAL P ³² THERAPY ADMINISTERED (MC.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST INJECTION (DAYS)	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED (ABOVE) BLOOD COUNTS IMMEDIATELY PRIOR TO DEATH (BELOW) ^b						% MONO- CYTES (MA- TURE)	% ORAN- ULOCYTES (POLYS AND STABS)	TO START OF P ³² (WK.)	TO DEATH (WK.)
					W.B.C. (PER C.M.M.)	R.B.C. ^c (MILLIONS PER C.M.M.)	HG (GM. PER 100 G.)	PLATE- LETS (PER C.M.M.)	% MONO- BLASTS AND YOUNG MONOCYTES	% MONO- CYTES (MA- TURE)				
1	40, F	None	0.142	2	49,000 90,000	2.11 1.82	5.9 5.6	8,000 40,000	84 86	2 6	0 0	0	14	14½
2	65, F	None	2.793	71	8,500 86,000	1.25 2.10 T-3	4.7 6.9	73,000 119,000	25 90	50 0	12 2	0	12	23
3	56, M	T	1.780	3	82,000 73,500	1.92 2.17	6.9 6.2	12,000 15,000	70 83	7 0	6 9	0	14	2
4	25, M	T	5.210	16	143,000 84,000	2.03 2.39 T-3	6.3 8.0	24,000 62,000	70 32	0 33	14 3	0	28	30½
5	53, F	None	11.056	62	18,200 11,300	1.56 1.59 T-16	5.5 4.4	58,000 10,000	31 97	19 1	1 0	0	4	11
6	69, F	T	4.964	8	87,500 169,000	2.30 2.86 T-1	7.8 7.9	69,000 320,000	94 93	3 4	1 0	0	12	14
7	62, M	None	4.504	4	72,500 100,000	1.72 1.10 T-1	6.4 4.4	20,000 11,000	91 90	2 0	0 0	0	6½	7
8	60, F	None	7.058	12	25,400 161,000	1.98 2.26 T-2	8.0 8.0	416,000 34,000	45 93	21 0	13 3	0	13	34
9	50, F	None	6.305	8	103,000 95,000	3.45 2.42	10.4 7.8	82,000 19,000	17 8	34 90	5 5	0	8	94
10	64, M	T	8.720	62	45,500 6,200	2.14 3.06 T-13	8.2 11.7	218,000 223,000	0 43	81 6	2 35	0	24	35+

^aT = Transfusion of whole blood.

^bPatient 10 was still living at the time this chart was prepared; the counts in these columns for Patient 10 are the last counts obtained eighty days after treatment was started.

^cT = Transfusions. The figure indicates the number of transfusions given.

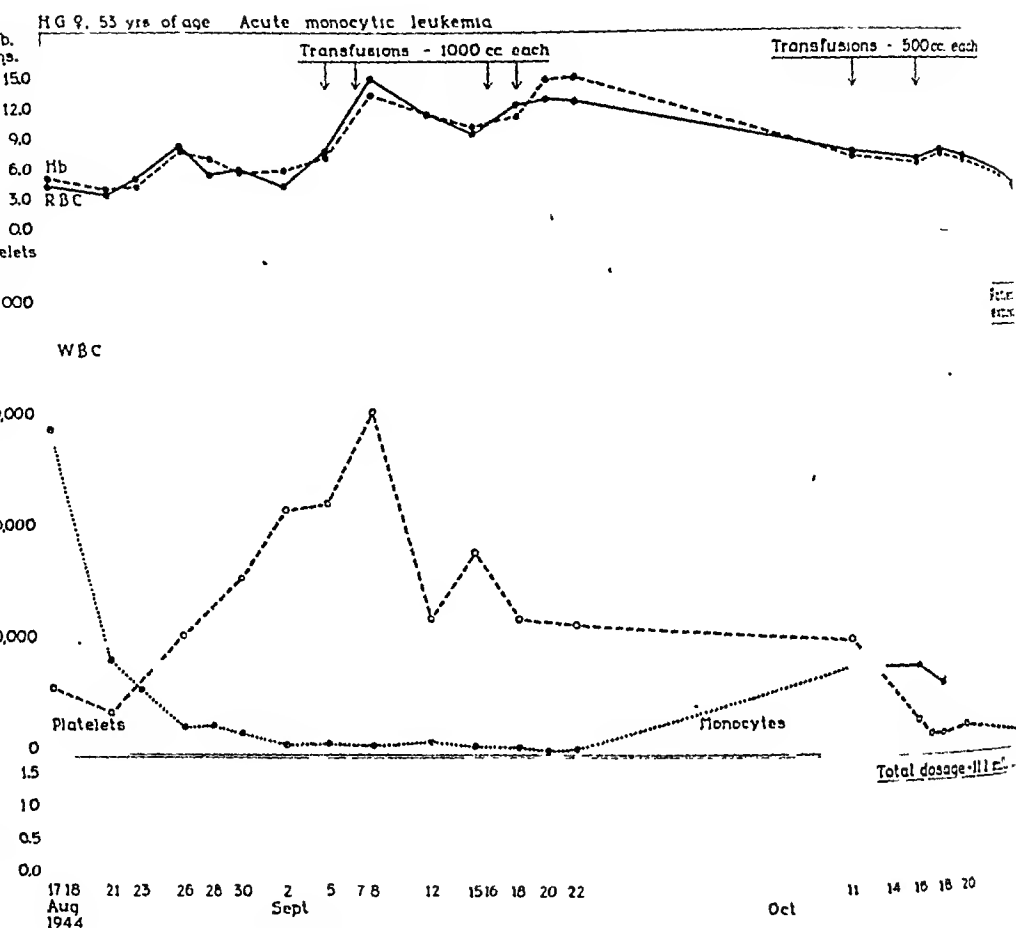


Fig. 14.—Monocytic leukemia. Response to P^{32} therapy.

Case Summary.—H. G., a housewife, 53 years of age, was in excellent health until July, 1944, at which time she developed pain in the lower jaw. The patient had many carious teeth and she attributed the pain to a toothache. However, within a few days the gums of both upper and lower jaws became swollen, red, and tender. Shortly thereafter she developed a sore throat, swelling of the glands in the neck, and fever. She began to have generalized headaches, spells of faintness, and increasing weakness. She was given sulfadiazine therapy (amount unknown) without improvement. In August, 1945, penicillin therapy was started; she was unimproved two days later when we first saw her.

Physical Examination: Blood pressure was 150/80. Patient was an obese, pale, acutely ill, white woman. There was marked hypertrophy of the gums with necrosis and ulceration, especially around the lower anterior teeth which were carious and loose. There was an elevated purplish area in the soft palate. The left anterior tonsillar pillar was ulcerated. The breath was foul. The submaxillary nodes were large, firm, and moderately tender; there was no other lymph node enlargement. There was an irregular area of hemorrhage in the right fundus. The liver and spleen were not palpable.

Laboratory Data: Blood counts (Aug. 17, 1945): leucocytes, 18,150; erythrocytes, 1,560,000; hemoglobin, 5.5 Gm.; reticulocytes, 0.5 per cent; platelets, 58,000; differential: stabs, 1 per cent; eosinophiles, 2 per cent; basophiles, 1 per cent; juveniles, 4 per cent; myelocytes, 30 per cent; lymphocytes, 9 per cent; mature monocytes, 49 per cent; immature

(Continued on opposite page.)

half. Furthermore, in all the patients who have died, the percentage of monocyte cells in the blood was greater just prior to death than at the time treatment was started. No conclusions can be drawn regarding the effect of P^{32} on the erythrocyte level since transfusions were given in all but three instances. The thrombocyte level increased in four patients, decreased in three, and showed no significant change in three. More detailed hematologic data on these patients are given in Table 15.

There was no definite evidence that any of these patients were benefited symptomatically by P^{32} therapy. Weakness temporarily became less severe in three instances (Patients 2, 5, and 10), but transfusions had been given to them repeatedly, and it seems likely that this was the factor responsible for the increase in strength. The two other most common symptoms were hemorrhagic manifestations (present in eight instances) and soreness of the gums (four instances). Both of these symptoms decreased in severity in Patient 5, but neither of them was significantly relieved in any of the other patients.

The physical signs likewise were not favorably influenced. At the time therapy with P^{32} was started, the lymph nodes were slightly enlarged in six patients, the spleen was palpable in five, and the gums were hypertrophied in four. In no instances was there any improvement in any of these abnormalities.

Hematologic data and the case report for Patient 5 are shown in Fig. 14. The response of this woman to therapy was not typical in that she lived longer than the average after treatment was started and showed somewhat more symptomatic improvement than did most of the patients in this group.

monocytes, 25 per cent; monoblasts, 6 per cent. A few normoblasts were seen in the peripheral blood. The sternal bone marrow differential (Aug. 17, 1944) was: stab, 3 per cent; juveniles, 8 per cent; "C" myelocytes, 9 per cent; "B" myelocytes, 4 per cent; immature monocytes, 52 per cent; monoblasts, 10 per cent; primitives, 13 per cent; plasma cells, 1 per cent. There were only 4 nucleated red blood cells per 100 myeloid cells. The urine was negative. The Kahn reaction was negative.

Course: Sulfadiazine and penicillin were discontinued on Aug. 15, 1944. On P^{32} therapy and transfusions she gradually improved. The temperature spiked daily to 39° C. until August 29, after which it dropped to lower levels. The ulcer of the tonsillar pillar healed and gums became much less sore; the hypertrophy and redness of the gums improved. On September 12 the temperature spiked to 40.4° C. and she developed a headache and marked restlessness. She became nauseated and vomited repeatedly. By September 22 these symptoms had subsided, and she felt so much stronger that she was discharged from the hospital. Two days after returning home she developed profuse vaginal bleeding and bleeding from the gums; this lasted several days. About October 1 she had severe epigastric pain followed by nausea and vomiting. The temperature began to rise. These symptoms persisted and she developed a rapidly spreading furunculosis over her back. She became semistuporous on October 17, and purpura and bleeding from the gums increased. She expired Oct. 27, 1944.

Post-mortem examination revealed extensive leukemic infiltration of the bone marrow, lymph nodes, lungs, gingivae, pancreas, liver, spleen, kidneys, adrenals, and uterus. The bone marrow was hyperplastic, the vast majority of the cells being monocytes; myeloid and erythroid elements were present but greatly decreased in numbers, and no megakaryocytes were seen. There were areas in the bone marrow in which the cells stained very faintly and were apparently undergoing early necrosis.

C. *Summary*.—Analysis of the hematologic and clinical data on our ten patients confirms the impression of previous investigators that radioactive phosphorus is of no value in the treatment of monocytic leukemia.

VII. HODGKIN'S DISEASE

A. *Review of the Literature*.—Four reports^{10, 12, 15, 17} have been published describing the results of therapy with radioactive phosphorus in thirty-eight patients with Hodgkin's disease. No case reports or data are given for any of these individuals except for the eleven patients studied by Hoster and Doan.¹⁷

Fitz-Hugh and Hodes¹⁰ treated five persons, only one of whom was improved following P^{32} therapy. This was a patient who was becoming x-ray-fast after two years of treatment. The other four patients were not benefited; three of them had received roentgen therapy previously, and the fourth patient had a huge mediastinal mass which did not respond to P^{32} but did regress following intensive x-radiation.

Low-Beer and co-workers¹² observed no satisfactory results in the nineteen patients they treated. Temporary improvement occurred only in those individuals who had mild symptoms of short duration, and in no case was the duration of remission more than one year.

Only one of the three patients treated with P^{32} by Warren¹⁵ was helped. All three had received x-ray therapy previously.

Of the eleven patients treated by Hoster and Doan,¹⁷ only five had been treated with x-radiation in the past. Radioactive phosphorus was given for from ten to thirty-seven weeks; the average biweekly dose was 2.3 millieuries. All eleven patients showed a decrease in the white blood cell level during the period of P^{32} therapy; the erythrocyte and platelet levels dropped in seven instances. In most cases these evidences of depression of bone marrow activity did not disappear after administration of P^{32} was stopped. Some of the patients developed additional lymph node enlargement during the period of treatment. Temporary benefit occurred in a few instances. X-radiation was given to nine of these patients after the cessation of P^{32} administration, and in every instance but one a favorable response was observed.

B. *Analysis of the Results Obtained in Treating Six Patients With Hodgkin's Disease*.—The six patients with Hodgkin's disease whom we have treated with radioactive phosphorus had had symptoms presumably referable to this disease for from eight to forty-two months. In each of them x-radiation had previously produced symptomatic improvement. Improvement following x-ray therapy had been least striking in Patient 1 of Table 16 (enlarged nodes in the neck decreased somewhat in size, and strength increased but the benefit was of only two weeks' duration); the greatest improvement following x-radiation was noted in Patient 4, who had experienced marked relief of all symptoms following six separate courses of roentgen therapy. All six of these patients are now dead.

The total dosage of P^{32} administered varied from 1.73 millieuries (given within five days) to 13.26 millieuries (administered during a period of seven months). The average total dosage was 8.62 millieuries.

Cytologic changes which occurred in the blood following therapy with P^{32} are shown in Table 16. In four of the six patients (Patients 1 to 4); P^{32} therapy was followed by depression of bone marrow activity, as manifested by significant decreases in the number of leucocytes, erythrocytes, and platelets in the peripheral blood. In two instances (Patients 2 and 3) the platelet count

TABLE 16. HEMATOLOGIC DATA ON SIX PATIENTS WITH HOPKIN'S DISEASE TREATED WITH RADIOACTIVE PHOSPHORUS

TABLE 16. HEMATOLOGIC DATA ON SIX PATIENTS WITH HODGKIN'S DISEASE										DURATION OF DIS- EASE FROM FIRST SYMPTOM			
PA- TIENT	AGE AND SEX	PRE- VIOUS TREAT- MENT ^a	TOTAL P ³² THERAPY ADMINIS- TERED (MC.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST INJEC- TION (DAYS)	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED (ABOVE) BLOOD COUNTS IMMEDIATELY PRIOR TO DEATH (BELOW) ^b								
					W.B.C. (PER C.M.M.)	R.B.C.s (MILLIONS PER C.M.M.)	Hg (GM. PER 100 G.G.)	PLATELETS (PER O.M.M.)	% MYELOID CELLS ^c	% LYMPHO- CYTES	% MONO- CYTES	TO START OF P ³² (MO.)	TO DEATH (MO.)
1	24, M	X-R	11.99	218	3,100 1,850	4.13 2.69	11.7 7.7	677,000 94,000	52 64	21 18	27 18	26	34
2	52, F	X-R	9.23	118	14,700 11,050	3.69 1.35	10.1 4.0	790,000 2,000	78 23	10 77	6 0	8	16
3	32, F	X-R	5.10	25	5,500 350	3.18 2.48 T-4	6.8 6.5	782,000 20,000	73 80	12 10	15 10	24	25½
4	29, M	X-R	13.26	229	4,050 3,450	4.16 3.18	12.6 11.0	715,000 235,000	79 72	12 22	9 6	42	59
5	18, F	X-R	1.73	5	9,600 9,300	4.53 4.65 T-11	10.5 11.2	861,000 690,000	85 92	9 4	6 4	15	17
6	27, M	X-R	10.41	301	9,800 11,250	3.75 3.83	12.4 9.5	855,000 1,140,000	92 91	1 3	7 6	36	50

^aX-R = X-ray.^bThe figures in the bottom row represent the blood counts just prior to death (if P³² treatment was continued until shortly before death), or one month after the last injection of P³² (if treatment was discontinued more than two months prior to death).^cT = Transfusions. The figure indicates the number of transfusions given (500 c.c. each).^dIncludes neutrophils, eosinophils, and basophilic cells of all stages of development.

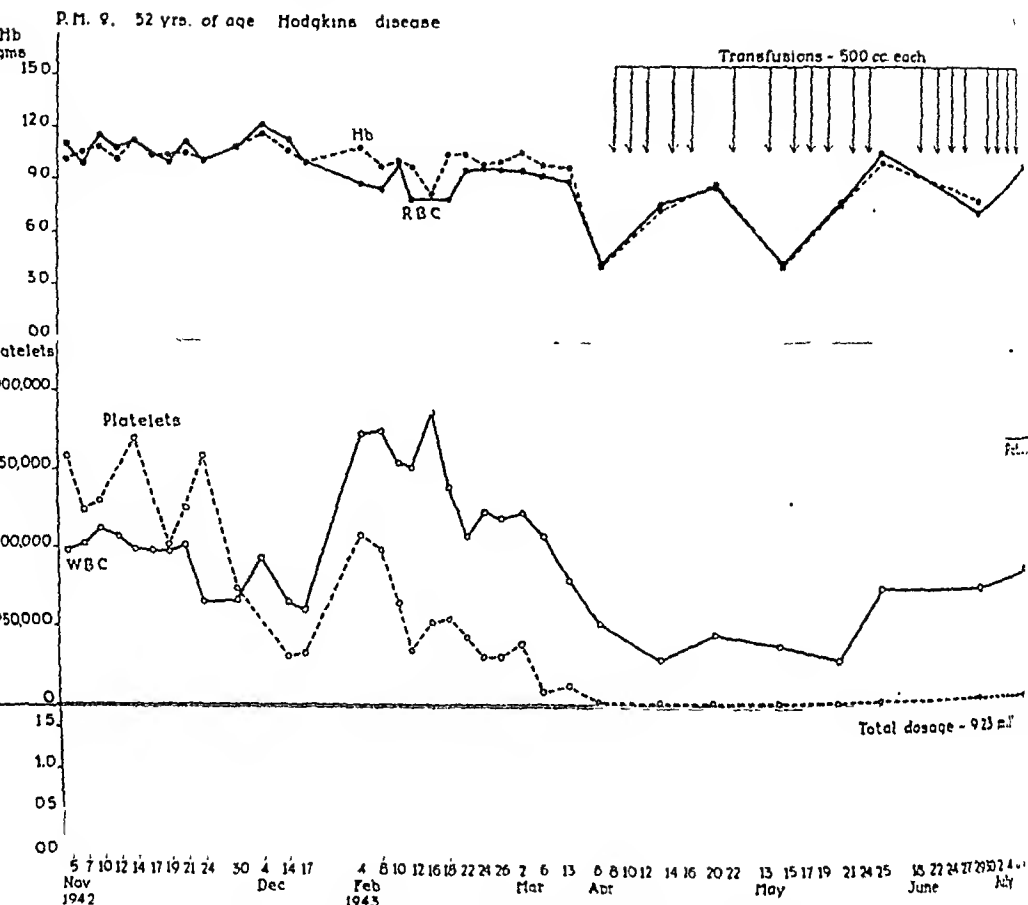


Fig. 15.—Hodgkin's disease. Hematologic changes following P^{32} therapy.

Case Summary.—P. M., a married woman 52 years of age, enjoyed excellent health until August, 1941, when she first noted a dry hacking cough. In May, 1942, the cough became productive of mucoid sputum. About this same time she began to have dull, aching pain in the left lower chest posteriorly. Shortly thereafter she began to have severe night sweats and increasing fatigue. She lost twelve pounds in weight. An x-ray of the chest in June, 1942, showed extensive pulmonary infiltration. Bronchoscopy revealed only chronic inflammation of the bronchi. By August, 1942, enlargement of the axillary and inguinal lymph nodes was apparent; a biopsy (right axillary node) revealed changes typical of Hodgkin's disease. She was given nine x-ray treatments to the right chest and axilla, following which the pain and cough disappeared. She gained nine pounds. During October the nodes in the left groin increased steadily in size and became sore and tender. Radioactive phosphorus therapy was started Nov. 5, 1942.

Physical Examination (Nov. 5, 1942): There was x-ray pigmentation over the right chest and back. A large matted mass of lymph nodes measuring 4 by 3 by 3 cm. was felt in the left inguinal region. Enlarged nodes were also felt in the neck and in the right axilla. The heart and lungs were normal. A firm mass thought to be spleen was felt in the left upper quadrant of the abdomen, but a distinct edge could not be outlined.

Laboratory Data: Urine was negative except for a faint trace of albumin. Kahn test was negative. Blood counts (Nov. 5, 1942): white blood cells, 14,700; red blood cells,

(Continued on opposite page.)

fell to extremely low levels, accompanied in the latter case by widespread subcutaneous hemorrhages. Patient 3 developed a leucopenia of 350 cells per cubic millimeter; a fatal bronchopneumonia then developed. Patients 5 and 6, who did not show these changes in the blood cytology, received such small doses of P^{32} that neither their symptoms nor physical signs showed any improvement following treatment.

Evaluation of the effect of P^{32} on the symptoms of Hodgkin's disease was difficult because of the notorious cyclic fluctuation in the severity of symptoms which occurs even when no treatment is given. Weakness was a prominent symptom in all six of these individuals; following therapy with P^{32} there was some increase in strength in four instances (Patients 1, 2, 4, and 6). The duration of this improvement varied from two weeks (Patient 6) to three months (Patient 2). Chills and fever, initially present in four instances (Patients 1, 3, 4, and 6) were eliminated only in Patient 1. Three patients (Patients 1, 5, and 6) complained of pain in the shoulders, chest, back, or flanks; the pain disappeared entirely in one (Patient 1) and became progressively worse in another (Patient 5). Cough, which was present in two patients (Patients 4 and 5), showed no improvement in either instance.

The physical signs were strikingly unaffected by P^{32} . Four patients (Patients 1, 2, 3, and 5) had enlarged lymph nodes, two (Patients 3 and 4) had splenomegaly, one (Patient 5) had hepatomegaly, and one (Patient 2) had edema of the ankles. None of these physical signs showed any measurable improvement following radiophosphorus therapy, except splenomegaly, which decreased in Patient 4, and lymph node enlargement which decreased in Patient 2.

Hematologic data and the case report for Patient 2 are shown in Fig. 15.

C. Summary.—The results obtained when these six patients were treated with P^{32} were similar to those which have been reported by other investigators. From this combined experience, it seems fair to conclude that P^{32} is inferior to x-radiation in the treatment of Hodgkin's disease. Even when the dosage of P^{32} employed is sufficiently large so that serious damage to the bone marrow results, enlarged lymph nodes and splenomegaly are relatively unaffected, and the infrequent symptomatic improvement which has been observed is of such

3,690,000; hemoglobin, 10.1 Gm.; reticulocytes, 2.8 per cent; platelets, 790,000; differential: eosinophiles 25 per cent; juveniles, 1 per cent; stabs, 4 per cent; segmented polymorphonuclear neutrophils, 48 per cent; lymphocytes, 16 per cent; and monocytes, 6 per cent. Sternal bone marrow examination showed 43 per cent eosinophiles, 39 per cent lymphocytes, 6 per cent plasma cells, and only 12 per cent cells of the myeloid series. Very few nucleated red blood cells were seen. There were many globules of fat present.

An x-ray of the chest showed considerable increase in the amount of infiltration in each lung.

Course: During the first four months of therapy there was little change in the patient's symptoms. The mass of lymph nodes in the left groin decreased somewhat in size. During March, she became progressively weaker, and by April 7 the extremities were covered with petechiae and ecchymoses. From that time she had constant hemorrhagic manifestations, and the erythrocyte level remained low in spite of twenty blood transfusions. She died eight months after P^{32} therapy was started. A post-mortem examination was not done.

transient duration as to be of no consequence. In only one case in our series was the symptomatic improvement following P^{32} as satisfactory or prolonged as the improvement which the same patient had previously experienced following x-ray therapy.

VIII. LYMPHOSARCOMA, RETICULUM CELL SARCOMA, AND GIANT FOLLICULAR LYMPHOBLASTOMA

A. *Review of the Literature.*—Kenney and his associates⁸ in 1942 published data on four patients with lymphosarcoma, two with reticulum cell sarcoma, and two with giant follicular lymphoblastoma treated with radioactive phosphorus. These patients had been given from 70 to 100 microcuries (0.07 to 0.1 millicuries) of P^{32} per kilogram of body weight in divided doses at intervals of from seven to fourteen days. Five patients (two with lymphosarcoma, two with reticulum cell lymphosarcoma, and one with giant follicular lymphoblastoma) had complete remissions for from one to eight months. One patient with lymphosarcoma had complete disappearance of neck nodes and 50 per cent decrease in the size of axillary nodes. Another individual with lymphosarcoma showed initial improvement followed by rapid increase in the size of the nodes. It was felt that this case represented a failure of radioactive phosphorus therapy, as the patient had shown disappearance of lymph node enlargement following small doses of x-ray therapy (300 roentgens) on three previous occasions. The second patient with giant follicular lymphoblastoma apparently had no enlarged lymph nodes, but the spleen was palpable 1 centimeter below the costal margin at the time P^{32} therapy was started; following treatment, the spleen became no longer palpable. These same cases were included in another report by Kenney.⁹

Two of the six patients with lymphosarcoma treated with P^{32} by Fitz-Hugh and Hodes¹⁰ had good remissions. One of these had become x-ray-fast; the clinical improvement which followed radioactive phosphorus therapy was still manifest six months later. Two of the patients were said to have been "benefited" for several months, following which both suffered relapses. One individual with reticulum cell sarcoma was not benefited by moderate doses of P^{32} ; he had previously received intensive roentgen radiation and had become "x-ray-fast." No data were recorded.

Low-Beer and co-workers¹² treated thirteen patients with generalized lymphosarcoma, seven of whom were dead at the time of the report. The initial response to therapy was satisfactory. Enlarged nodes usually disappeared, and the general condition of the patients was improved, in some cases returning to normal. The authors made the interesting statement that, "In no patient treated for lymphosarcoma, even though it has been found necessary to build up a high radiation level, has the peripheral blood picture changed appreciably." A case history was given for one of these individuals, a woman, 66 years of age; whose symptoms were of six months' duration. She had received no previous therapy of any sort. She was given thirteen doses of P^{32} , totaling 14.1 millicuries, in a period of nine weeks. By the time the course of therapy was completed, she was asymptomatic; enlarged lymph nodes and abdominal masses were no longer palpable. The remission lasted almost eleven months, at the end of which time a second course of therapy again resulted in complete remission. The leucocyte and erythrocyte levels in the peripheral blood were practically unaffected by the treatments.

Kenney and Craver¹³ analyzed the results of treatment of eight patients, but five of these had been previously reported.⁹ The three new patients all had reticulum cell sarcoma; they were given nineteen doses of P^{32} totaling 16.8 millicuries in a period of three months, ten doses totaling 8.84 millicuries in a period of three weeks, and thirty-five doses totaling 22.8 millicuries in two months time, respectively. The first two patients did not improve, and enlarged lymph nodes remained the same or increased in size. The third individual showed complete regression of skin nodules and enlarged lymph nodes. In all three instances the erythrocyte level dropped significantly following therapy, and the leucocyte and platelet level decreased in the two cases where these determinations were done.

TABLE 17. HEMATOLOGIC DATA ON FIVE PATIENTS WITH LYMPHOSARCOMA, THREE PATIENTS WITH RETICULUM CELL SARCOMA, AND ONE PATIENT WITH GIANT FOLLICULAR LYMPHOBLASTOMA TREATED WITH RADIOACTIVE PHOSPHORUS

PATIENT	AGE AND SEX	PREVIOUS TREATMENT ^a	TOTAL P ³² THERAPY ADMINISTERED (MC.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST INJECTION (DAYS)	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED (ABOVE) BLOOD COUNTS ONE MONTH AFTER LAST TREATMENT, OR IMMEDIATELY PRIOR TO DEATH (BELOW)							TO START OF P ³² (MO.)	TO DEATH (MO.)
					W.B.C. (PER C.M.M.)	R.B.C. ^b (MILLIONS PER C.C.)	Hb (GM. PER 100 C.C.)	PLATELETS (PER C.M.M.)	% MYELOID CELLS	% LYMPHO-CYTES	% MONO-CYTES		
<i>Lymphosarcoma</i> L ₁	46, F	X-R	3.72	12	9,650 6,950	4.38 4.32	11.0 11.3	1,010,000 553,000	79 83	6 8	15 9	24	1
	L ₂	X-R	3.01	129	3,400 4,400	4.15 3.42	12.2 11.2	656,000 308,000	70 54	14 27	16 19	15	29
	L ₃	None	0.29	30	5,200 2,350	4.42 4.04	14.9 15.0	972,000 350,000	80 72	7 14	13 14	3	19+
	L ₄	X-R	6.24	159	9,750 5,000	4.30 4.29	12.2 13.3	1,100,000 526,000	78 73	9 17	13 10	12	39+
	L ₅	X-R	7.83		6,400 2,150	4.93 4.50	13.0 12.4	454,000 45,000	57 68	31 19	12 13	17	21½
<i>Reticulum cell sarcoma</i> R ₁	59, M	None	10.86	135	11,400 9,000	3.45 3.82	11.3 12.5	649,000 266,000	85 90	8 3	7 7	1	5½
	R ₂	X-R	15.08	127	4,150 1,100	3.51 1.08	10.6 4.2	667,000 4,000	77 40	8 24	15 36	5	9½
	R ₃	T	3.00	15	3,250 2,550	3.99 3.55	14.1 11.9	439,000 348,000	42 6	40 24	11 ^c 0 ^d	5	6½
<i>Giant follicular lymphoblastoma</i> F ₁	13, M	X-R	5.9	53	3,200 1,500	4.76 4.31	15.5 14.7	762,000 232,000	60 43	36 48	4 9	36½	75+

^aX-R = X-ray; T = transfusions.

^bT = Transfusions. The figure indicates the number given.

^cSeven per cent of the leucocytes in the peripheral blood at this time were reticulum cells.

^dSeventy per cent of the leucocytes in the peripheral blood at this time were reticulum cells.

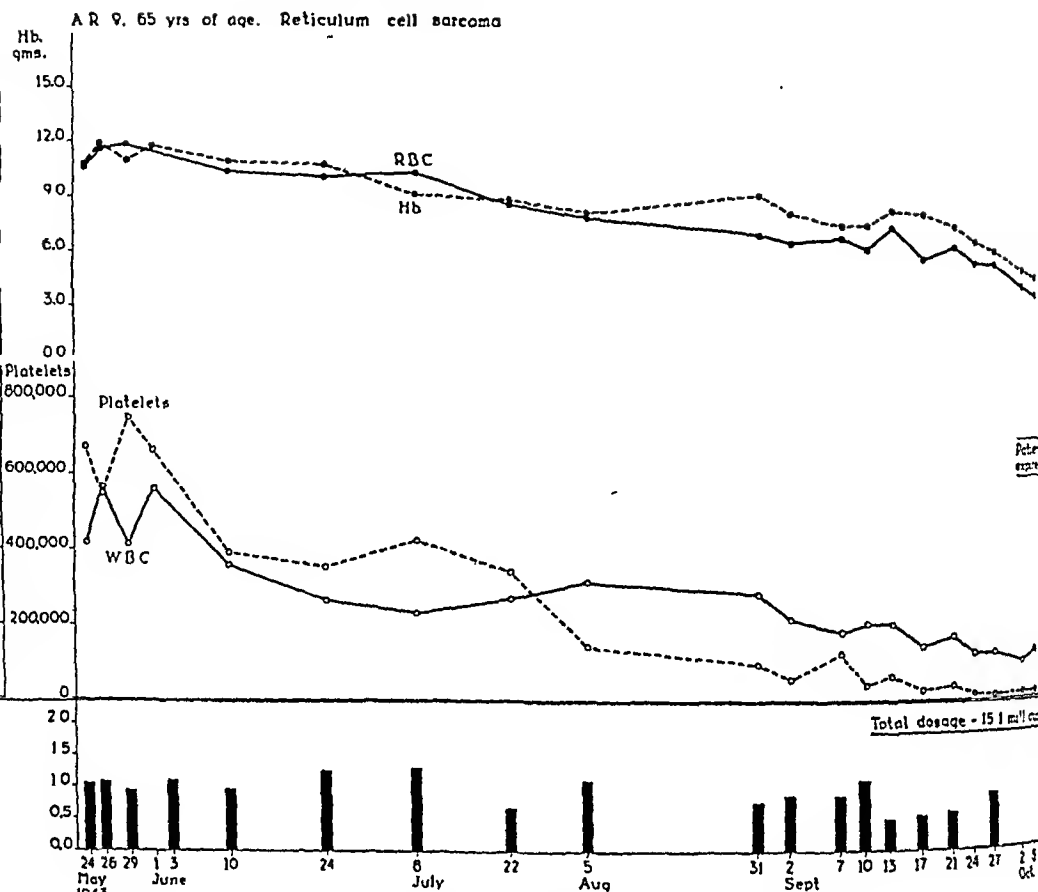


Fig. 16.—Reticulum cell sarcoma. Effect of P^{32} therapy on the blood.

Case Summary.—A. R., a woman 65 years of age, first noted a lump in the right axilla in December, 1942. Because the mass increased rapidly in size, it was removed in January, 1943. Histopathologic examination established the diagnosis of reticulum cell sarcoma. One month later a mass appeared in the right breast; this subsided promptly following x-ray therapy (1,600 roentgens). Early in May, 1943, a mass suddenly developed in the left side of the neck. Following x-ray therapy (1,600 roentgens) this mass decreased in size but did not disappear. During May, 1943, the patient noticed increasing fatigability, dyspnea, and cough.

Physical Examination (May 21, 1943): Blood pressure was 150/95. There was x-ray pigmentation over the neck and anterior thorax. Several rubbery nodes about 1 cm. in diameter were felt in the neck. A firm mass 5 by 6 cm. in size was felt in the right breast. Moist râles were heard at the extreme left base. There was tenderness in the lower abdomen, but no masses were felt, and the liver and spleen were not palpable.

Laboratory Data: Blood counts (May 24, 1943): leucocytes, 4,100 per cubic millimeter; erythrocytes, 3,510,000; hemoglobin, 10.6 Gm.; reticulocytes, 0.4 per cent; platelets, 667,000; differential, normal. Urine was negative. Kahn reaction was negative.

Course: After administration of P^{32} was begun on May 24, 1943, there was little change in patient's weakness and cough. Her appetite remained poor, and she felt drowsy. On May 30, 1943, a small mass about 2 by 3 cm. in size was felt deep in the abdomen just below the umbilicus. Thereafter this mass increased steadily in size until by Aug. 5, 1943,

(Continued on opposite page.)

None of the four patients with lymphosarcoma treated with P^{32} by Warren¹⁵ were helped. Two of them had had no previous therapy and the other two had received x-radiation in the past. No other data were given.

B. Analysis of the Results Obtained in Treating Five Patients With Lymphosarcoma, Three Patients With Reticulum Cell Sarcoma, and One Patient With Giant Follicular Lymphoblastoma.—The duration of symptoms in the group of eight patients with lymphosarcoma or reticulum cell sarcoma varied from one to twenty-four months (Table 17). Five of these individuals had had previous x-ray therapy. In Patients L1 and L4 there were no symptoms at the time x-radiation was administered and the only manifestation of disease was enlarged nodes; these disappeared promptly and completely after the treatments. Patient R2 also had no symptoms; a mass in the right breast disappeared completely after her first course of roentgen radiation, but enlarged cervical nodes underwent only partial regression following a subsequent course of x-ray therapy. Marked weakness, the only complaint of Patient L5, was entirely relieved by x-ray. Patient L2 had no symptoms, and the only tumor mass was removed surgically just prior to x-radiation. Thus, therapy cannot be evaluated in this instance.

The total dosage of P^{32} administered varied from 1.1 to 15.1 millieuries, with an average of 7.0 millieuries. In spite of the relatively small dosage employed in most cases, the leucocyte and thrombocyte levels in the blood decreased in all instances, and the erythrocyte level decreased by more than 400,000 cells per cubic millimeter in four cases. The platelet count of Patient R2 dropped to almost zero and she died following massive hemorrhage into the intestines and peritoneum.

Administration of radioactive phosphorus was followed by significant clinical improvement in only two instances. When treatment was started, Patient R1 had pain in the back, frequent night sweats, dyspnea on exertion, profound weakness, and marked anorexia. One month later all of these symptoms had completely disappeared except weakness and anorexia, which had improved. However, in addition to P^{32} , the patient had received five blood transfusions. The clinical remission lasted about three months. Patient L5 had no complaints other than weakness; within two weeks after P^{32} therapy was instituted he felt much stronger, and this symptomatic remission lasted about four months. In all other patients, not only was there no clinical improvement, but symptoms became progressively more severe.

it measured 10 by 15 cm.; it was rubbery and nodular. The cervical nodes increased in size, and axillary and inguinal nodes appeared. Pitting edema of the back and legs became progressively more marked. By Oct. 6, 1943, the patient was semicomatose, the temperature was 38.5° C., and the abdomen was filled with nodular masses and fluid. She expired five months after therapy with P^{32} had been started.

At post-mortem examination, the patient was found to have reticulum cell sarcoma involving the mediastinal, mesenteric, periaortic, peripancreatic, and iliac lymph nodes; these latter nodes had fused to form tremendous nodular masses. There was sarcomatous involvement of the mucosa of the duodenum and the stomach. Blood was found in the intestine and in the peritoneal cavity (1,500 c.c.).

All of these patients except one had palpable or roentgenologically demonstrable enlargement of lymph nodes. In three instances (Patients R1, R3, and L5) the nodes decreased significantly in size following P^{32} therapy, whereas in the other four they increased progressively in size. The spleen was initially palpable in only two instances (Patients L4 and L5); in Patient L4 progressive enlargement occurred, while in Patient L5 the lower pole of the spleen, which originally measured 23 centimeters below the costal margin in the mid-clavicular line, measured only 12 centimeters two months later. In no case was the regression in size of the lymph nodes or spleen of more than a few months' duration.

Hematologic data and the case report for Patient R2 are shown in Fig. 16.

One patient with giant follicular lymphoblastoma (Brill-Symmer's disease) has been treated. This was a boy, 13 years of age, who had had one slowly enlarging nodule in his neck for three years, and several other cervical nodes for about six months. His mother insisted he had tired easily for two to three years, but the boy denied this. There were no abnormal physical signs other than moderate enlargement of the cervical and axillary nodes. The blood counts were normal in all respects. Biopsy of a cervical node revealed the typical microscopic appearance of giant follicular lymphoblastoma. During a period of seven months he was given thirteen injections of P^{32} totaling 5.91 millieuries (Table 17). At the end of this time there were no palpable nodes, and the patient played on his school basketball team. During the course of treatment, the leucocyte level dropped as low as 1,500 and did not return to normal until a year later; the other cellular elements in the blood were unaffected. About eighteen months after completion of the course of treatment several palpable cervical and axillary nodes reappeared. One of the cervical nodes was biopsied; the sections did not show the changes of giant follicular lymphoblastoma but only scattered areas of fibrosis. The patient remains asymptomatic, and the blood counts are all normal two years after the first treatment.

C. Summary.—There are several reports in the literature which suggest that P^{32} may be of value in the treatment of lymphosarcoma, reticulum cell sarcoma, and giant follicular lymphoblastoma. Twenty-one patients have been treated and followed for a sufficient length of time so that it was possible to draw at least tentative conclusions regarding the effect of the therapy; in six patients complete regression of all symptoms and signs of disease was observed, in four patients partial regression occurred, and in eleven there was no detectable improvement. In no instances had the remission lasted more than eleven months at the time of the report. Radioactive phosphorus therapy was considered a failure in all of the patients with lymphosarcoma and reticulum cell sarcoma treated at the Mallinckrodt Institute of Radiology. Relief of symptoms occurred in only two patients, in one of whom five transfusions probably accounted for the improvement, and, in the other, increase in strength was the only evidence of benefit. Enlarged lymph nodes decreased in size in three instances, and an enlarged spleen became smaller in one instance. The cellular elements of the blood decreased significantly in number following treatment. In conclusion, it is felt that the unfavorable influence of P^{32} therapy on the bone mar-

row more than offsets the occasional clinical benefit which may occur following such treatment. It is our impression that x-radiation is a more effective and less dangerous form of therapy than P^{32} for this group of diseases.

IX. MULTIPLE MYELOMA

Under this heading are included a few cases in which plasma cells were found in the peripheral blood and bone marrow in large numbers. Some authors have labeled these as plasma cell leukemia and probably would have made a similar diagnosis in Patient 4 in our series (Table 18). This man had typical multiple myeloma with numerous characteristic x-ray lesions in the skull, pelvis, humerus (pathologic fracture also present), and left femur; Bence-Jones proteinuria was present; 28 per cent of the cells in the bone marrow were plasma cells; and a biopsy of the lesion of the left humerus taken at the time of open reduction of the fracture showed multiple myeloma. The diagnosis was confirmed by post-mortem examination. When this patient was first observed there were no plasma cells in the peripheral blood, but during the course of P^{32} therapy they appeared in the blood in increasing numbers and finally accounted for 20 per cent of all the leucocytes at the time of death. All cases will be discussed together without differentiation as to whether myeloma cells were found in the peripheral blood.

A. Review of the Literature.—There are four reports^{5, 10, 12, 13} which describe the results of therapy with radioactive phosphorus in seventeen patients with multiple myeloma or plasma cell leukemia. Case reports are given for only three of these individuals.

Erf and associates⁵ treated a man, 59 years of age, who had weakness and aching pains of two weeks' duration. There were many plasma cells in the blood and bone marrow and large amounts of Bence-Jones protein in the urine. The diagnosis of acute plasmacytoid leukemia was made, and 14.9 millicuries of P^{32} were administered orally in nineteen days. Death occurred two months after treatment was started; post-mortem examination revealed plasma cell infiltration of most organs including the bone marrow, kidneys, and submucosa of the gastrointestinal tract.

Fitz-Hugh and Hodes¹⁰ state that the one patient with multiple myeloma whom they treated with P^{32} was not benefited by the drug. No data were given.

Eleven patients were treated by Low-Beer and co-workers.¹² The authors stated that at the time of the report the response of these individuals to P^{32} therapy had not been uniform. In some patients there had been marked relief of pain and a restoration to almost normal activity. However, no appreciable change in the roentgenographic appearance of the lesions had occurred. A case report was given for one patient, a man 49 years of age, who had been found to have multiple myeloma fifteen months earlier. Previous x-ray therapy to the spine and left leg had afforded partial relief from pain. During a period of five months, 5.0 millicuries of P^{32} had been given orally and 13.8 millicuries intravenously. The patient's physical activity had been very limited but several months after the first dose of P^{32} he became able to walk and drive his automobile. Without giving any specific data, the authors state that, "There was no adverse change in the blood levels during or after treatment."

Three patients with "plasmacytoma" and one with plasma cell leukemia were treated with P^{32} by Warren.¹⁵ Two of the patients with plasmacytoma were said to have been helped by the treatment. A case report was given for one patient, a woman 66 years of age, who had had symptoms for about one year. X-ray studies showed lesions throughout the whole skeleton; 6 per cent of the leucocytes in the peripheral blood were plasma cells; and the sternal marrow contained masses of plasma cells. Between Nov. 12, 1941, and April 14, 1942,

TABLE 18. HEMATOLOGIC DATA ON EIGHT PATIENTS WITH MULTIPLE MYELOMA TREATED WITH RADIOACTIVE PHOSPHORUS

PA-TIENT AGE AND SEX		PREVI- OUS TREAT- MENT ^a	DATA UPON WHICH DIAGNOSIS WAS BASED ^b	TOTAL P ³² THERAPY ADMINIS- TERED (MG.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST IN- JECTION (DAYS)	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED (ABOVE) OR IMMEDIATELY PRIOR TO DEATH (BELOW)										DURATION OF DISEASE FROM FIRST SYMPTOM	
						W.R.C. (PER C.M.M.)	R.R.C. ^c (MILLIONS PER C.M.M.)	Hg (GM. PER 100 C.C.)	PLATE- LETS (PER C.M.M.)	% MYELOID CELLS	% LYMPHO- CYTES	% MONO- CYTES	% PLASMA CELLS	TO START OF P ³² (MO.)	TO DEATH (MO.)		
1	47, F	X-R	B, M(7%), X, P	16.35	1096	4,550	2.61	11.2	808,000	74	21	5	0	36	62		
2	66, M	None	M(14%), G, A	5.88	35	4,300	2.95 T-12	9.7	348,000	77	12	11	0	12?	20		
3	62, F	None	B, M(90%), X, A	8.66	32	7,200	4.04	12.9	582,000	71	23	6	0	12	14		
4	49, M	None	B, M(28%), X, P, A	5.00	14	3,500	3.67	12.7	338,000	66	26	8	0	12	10		
5	53, F	None	B, M(46%), X, P, A	10.91	110	11,000	2.29	8.0	550,000	83	12	5	0	12	14		
6	69, F	LE	M(48%), X, P, G	4.89	21	1,500	1.33 T-3	4.5	54,000	90	0	10	0	9	10		
7	44, M	None	X, P, G	16.75	253	7,500	4.19	11.6	1,307,000	66	22	11	3	36	41		
8	58, M	None	M(83%), X, G	8.63	145	10,050	2.48	11.8	1,518,000	72	5	15	8	36?	38		
						1,900	2.02 T-3	5.7	600,000	46	31	6	3	36	41		
						4,000	1.80	6.3	12,000	30	26	7	2	36?	38		
						2,400	1.79 T-3	6.3	640,000	65	24	4	2	12	21+		
						10,800	4.48	13.1	910,000	70	16	7	0	12	21+		
						9,500	3.34 T-3	12.4	354,000	78	15	7	0	12	21+		
						6,400	2.00	8.1	224,000	36	52	10	2	9	17+		
						3,350	2.50 T-5	10.1	20,000	32	59	8	1				

^aX-R = X-ray; LE = liver extract.
^bEach letter listed indicates that the corresponding test or examination was positive for multiple myeloma. B = Biopsy of tumor mass; G = Gallbladder; A = Aorta; X = X-ray; P = Bone-marrow; M = Bone-marrow; L = Liver; S = Spleen; K = Kidney; T = Testis; O = Ovary; U = Uterus; V = Vagina; C = Cervix; N = Nose; E = Ear; H = Heart; L = Lung; S = Skin; M = Mouth; N = Neck; T = Throat; O = Oral cavity; U = Urinary tract; V = Vascular system; C = Central nervous system; P = Peripheral nervous system; K = Kidney; T = Testis; O = Ovary; U = Uterus; V = Vagina; C = Cervix; N = Nose; E = Ear; H = Heart; L = Lung; S = Skin; M = Mouth; N = Neck; T = Throat; O = Oral cavity; U = Urinary tract; V = Vascular system; C = Central nervous system; P = Peripheral nervous system.

*X-R = X-ray; LE = liver extract.

Each letter listed indicates that the corresponding test or examination was positive, or that the results were compatible with the diagnosis of multiple myeloma. B = Biopsy of tumor mass; M = percentage of plasma cells in sternal marrow; X = x-ray films of skeleton including skull; P = Bence-Jones proteinuria; G = elevated serum globulin; A = autopsy.

^aT = Transfusions. The figures indicate the number given.

she received 12.1 millicuries of P^{32} intravenously. Bone pain disappeared promptly after treatment was started, and five months later x-ray examination showed some recalcification of the osteolytic foci. The erythrocyte and leucocyte levels were both within normal limits at this time. Between Sept. 24, 1942, and July 31, 1943, she received an additional 9.67 millicuries of P^{32} . Her clinical course was complicated by the development of a duodenal ulcer. She was transfused repeatedly. In November, 1943, she developed lobar pneumonia from which she died; the leucocyte count at this time was 1,550, and the erythrocyte count was 1,630,000. The author felt that this patient showed appreciable improvement lasting from November, 1941, to February, 1943.

B. Analysis of the Results Obtained in Treating Eight Patients With Multiple Myeloma With Radioactive Phosphorus.—The diagnosis of multiple myeloma in all eight patients included in this study was accepted only after myeloma cells were found in the bone marrow. Four of the patients had tumor masses which were biopsied and which showed the characteristic histopathologic changes of multiple myeloma. Supporting evidence for the diagnosis was obtained from chemical and roentgenologic studies (see column 4 of Table 18). Necropsy has been performed on four of the patients.

The duration of symptoms at the time therapy with P^{32} was started varied from nine to thirty-six months, with an average of twenty months. Only one patient (Patient 1) had received previous radiation therapy; she had had severe pain in the spine which was markedly relieved by x-radiation.

The total dosage of P^{32} administered ranged from 5.0 to 16.7 millicuries, with an average of 9.6 millicuries. The leucocyte level decreased to below 4,000 cells per cubic millimeter in five instances and below 2,000 in two instances; the thrombocyte level fell to 100,000 per cubic millimeter or less in four patients (Table 18). The leucocyte count in Patient 3 dropped as low as 550 cells per cubic millimeter three weeks prior to death and fluctuated thereafter between 800 and 2,100; bilateral pneumonia with septicemia due to hemolytic *Staphylococcus aureus* was the immediate cause of death. In Patient 5, the leucocyte level ranged between 1,400 and 2,300 cells per cubic millimeter during the last three weeks of life. She developed a meningitis and a bacteremia (pneumococcus type 29); this infection was not controlled even with intensive therapy with penicillin and sulfadiazine. Platelets virtually disappeared from the blood during the last two months of this patient's life; she developed extensive petechiae and ecchymoses and a terminal subarachnoid hemorrhage. There can be little doubt but that depression of leucocyte and platelet formation in the bone marrow by the P^{32} was the underlying factor responsible for death. The effect of radioactive phosphorus on erythrocytogenesis in these patients could not be estimated accurately because all but two of them received repeated blood transfusions.

Treatment with radioactive phosphorus was followed by clinical improvement in only two instances. When therapy was started, Patient 8 (Table 18) had no symptoms except dyspnea and marked weakness. One month later he felt much stronger and dyspnea had disappeared. It was felt that this improvement was due primarily to five blood transfusions which he had received during the course of P^{32} therapy. The other patient who improved (Patient 6) also received transfusions (three) which might have accounted for increase in

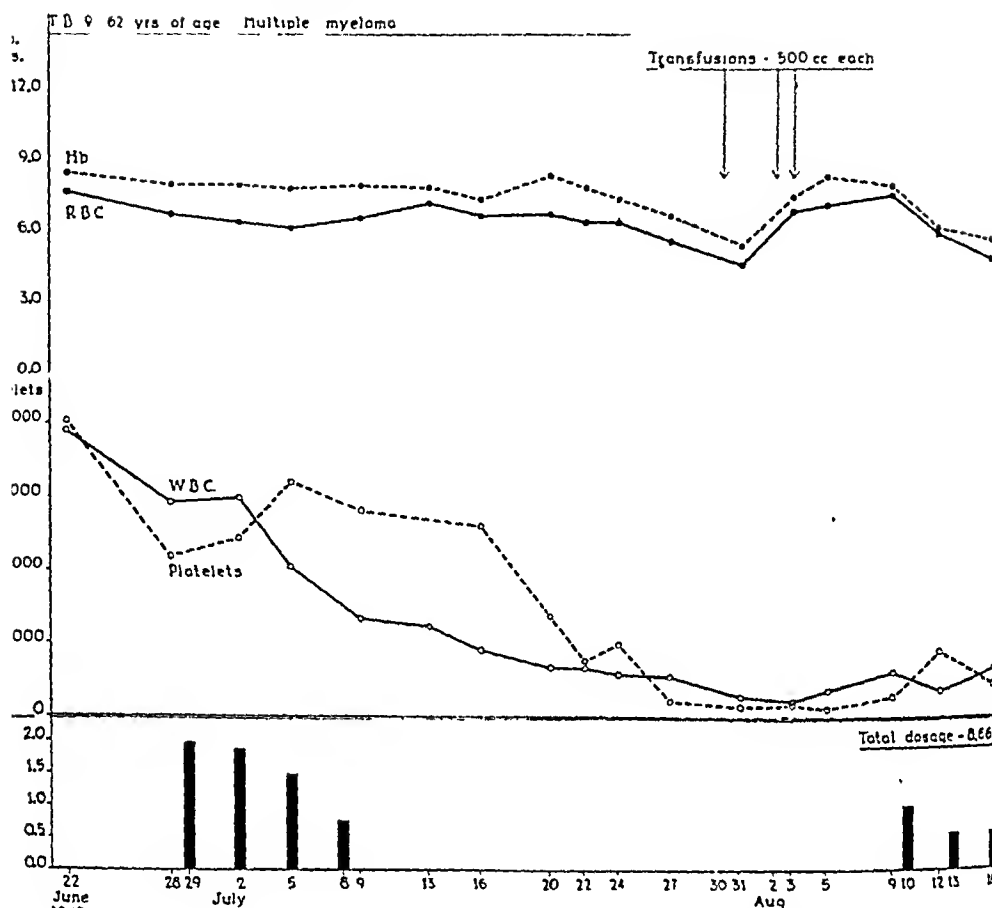


Fig. 17.—Multiple myeloma. Hematologic changes following P^{32} therapy.

Case Summary.—T. B., a married woman, 62 years of age, had an attack of pain in her shoulders and spine lasting two weeks in June, 1942. She then felt perfectly well until February, 1943, when her legs became weak; this was followed first by numbness and then by paralysis of the legs. The paralysis gradually ascended, and by April, 1943, she was totally paralyzed from the waist down. During June she developed blurring of vision, diplopia, and a coarse tremor of the hands.

Physical Examination (June 28, 1943): Blood pressure was 200/100. The patient was moderately obese. Her back was stiff, and there was tenderness to percussion over the upper dorsal vertebrae. The bladder was distended to the umbilicus. Complete flaccid paralysis of both lower extremities was present together with some weakness and spasticity of the upper extremities. There was complete sensory loss below the level of the sixth dorsal segment. Abdominal, knee jerk, and ankle jerk reflexes were absent bilaterally.

Laboratory Data: Urinalysis was negative, no Bence-Jones protein was present. The Kahn reaction was negative. Total plasma proteins were 6.2 Gm. per cent, with albumin 3.1 Gm., and globulin 3.1 Gm. Basal metabolic rate was +15 per cent. Blood counts (June 22, 1943): leucocytes, 14,650; erythrocytes, 2,580,000; hemoglobin, 8.5 Gm.; reticulocytes, 2.8 per cent; platelets, 1,050,000; differential, normal. Sternal bone marrow examination (June 29,

(Continued on opposite page.)

strength. However, in addition, she also experienced marked relief of pain. Prior to P^{32} therapy she had severe pain in the back and flanks, hands, shoulders, and the right knee. One month later these pains had entirely disappeared. The patient said she had had arthritis for thirty years, and an x-ray of the knee showed osteoarthritic changes. X-rays of the spine showed compression fractures of the ninth and tenth dorsal vertebrae. Thus, it is difficult to evaluate how much, if any, of the relief of pain could be attributed to the therapy with radioactive phosphorus.

No improvement in physical signs occurred in any of these individuals. In one instance (Patient 1) it was felt that x-rays of the skeleton taken after treatment when compared with earlier films showed some evidence of repair proceeding in the bone lesions as evidenced by stationary size, a tendency to demineralization, and sharper outline of the defects by deposits of dense bone in their margins.

One patient (Patient 4) developed uremia. On admission to the hospital his plasma nonprotein nitrogen level was 31 milligrams per cent, and the uric acid level was 7.3 milligrams per cent. Two weeks later, after having received 5.0 millicuries of P^{32} , he was disoriented; the nonprotein nitrogen value had risen to 180 milligrams per cent, and the uric acid to 16.6 milligrams per cent. It was felt that rapid necrosis of tumor cells as a result of radioactive phosphorus administration might have contributed to the azotemia. At post-mortem examination, the tubules of both kidneys were found to be blocked with deposits of urates.

Hematologic data and the case report for Patient 3 are shown in Fig. 17.

C. Summary.—Radioactive phosphorus has not proved to be a valuable therapeutic agent for the treatment of multiple myeloma. In no case has there been any convincing objective evidence of improvement following treatment. Low-Beer and associates¹² and Warren¹⁵ reported relief of pain in a few patients; Patient 6 in our series experienced considerable relief of pain, but the causal relationship of this improvement to P^{32} was obscure. It is our impression that radiophosphorus therapy significantly shortened the duration of life in two patients by producing severe leucopenia and thrombocytopenia.

1943) showed: plasma cells, 90 per cent; myeloid cells, 8 per cent; primitive cells, 1.5 per cent; and nucleated red blood cells, 0.5 per cent.

X-ray films revealed lesions typical of multiple myeloma in the skull, ribs, and spine.

Course: Three weeks after P^{32} therapy was started the patient developed cellulitis of the buttocks; this progressed in spite of sulfadiazine therapy. A week later a purpuric skin lesion appeared on the shoulders. Terminally, she developed bilateral bronchopneumonia, and a blood culture was positive for hemolytic *Staphylococcus aureus*. She expired fifty-three days after treatment was started.

A second sternal marrow aspiration performed on the thirty-sixth day of P^{32} therapy revealed a hypercellular marrow; 93 per cent of the cells were plasma cells, 4 per cent were nucleated red blood cells; no megakaryocytes were seen.

Post-mortem examination revealed plasma cell myeloma involving the marrow of all bones examined (vertebrae, ribs, and sternum), collapse of the sixth thoracic vertebra, multiple abscesses in the myocardium, kidneys, and vertebrae, bronchopneumonia with abscess formation of all lobes of the lungs, and hemorrhagic cystitis of the urinary bladder.

TABLE 19. HEMATOLOGIC DATA ON EIGHT PATIENTS WITH VARIOUS DISEASES TREATED WITH RADIOACTIVE PHOSPHORUS

PA- TIENT	AGE AND SEX	PREVIOUS TREAT- MENT ^a	DIAGNOSIS	TOTAL P ³² THERAPY ADMINIS- TERED (MG.)	DURATION OF P ³² THERAPY FROM FIRST INJECTION TO LAST INJEC- TION (DAYS)	BLOOD COUNTS AT TIME P ³² THERAPY WAS STARTED (ABOVE) BLOOD COUNTS ONE MONTH AFTER LAST TREATMENT, OR IMMEDIATELY PRIOR TO DEATH (BELOW)						DURATION OF DISEASE FROM FIRST SYMPTOM		
						W.B.C. (PER C.M.M.)	R.B.C. ^b (MIL- LIONS PER C.M.M.)	HG (GM. PER 100 G.C.)	PLATE- LETS (PER C.M.M.)	% MYELOID CELLS	% LYMPHO- CYTES	% MONO- CYTES	TO START OF P ³² (MO.)	TO DEATH (MO.)
1	20, M	Sur	Ewing's tumor	5.3	8	15,050	4.70	14.7	1,130,000	64	16	20	3	?
2	9, F	None	Ewing's tumor	3.1	18	10,600	4.46	14.1	1,600,000	68	10	22	2	?
3	17, M	X-R	Ewing's tumor	11.5	53	7,200	3.79	9.2	857,000	79	15	6	2	5
4	18, F	None	Ewing's tumor	7.7	26	5,700	3.48	9.2	835,000	76	11	13	2	5
5	52, M	None	Malignant mel- anoma	12.9	288	10,000	4.59	10.7	421,000	70	15	15	4	5
6	55, M	FT	Anaplastic ear- chima	18.4	549	1,950	0.78	2.2	65,000	78	11	11	6	28+
7	38, F	X-R, FT	Mycosis fun- goides	15.0	111	7,850	2.84	8.0	485,000	85	4	11	18	54+
8	1, M	None	Mycosis fun- goides Xanthomatosis	0.33	4	3,100	1.45	3.8	190,000	74	14	12	7	74
						5,950	4.10	11.8	1,390,000	64	28	8		
						3,250	4.13	11.7	326,000	53	26	21		
						7,100	4.57	15.4	1,080,000	87	8	5		
						4,570	3.75	12.0	367,000	84	8	8		
						8,600	4.45	15.0	1,110,000	78	7	15		
						6,550	4.23	10.7	711,000	77	13	10		
						6,050	3.10	7.9	127,000	44	48	8		
						11,150	4.75	13.8	38,000	68	30	2		

^aSur = Surgery; X-R = x-ray; FT = fever therapy.^bT = Transfusions. The figure indicates the number given.

X. MISCELLANEOUS DISEASES

A. *Review of the Literature.*—Eight patients with osteogenic sarcoma, one with metastasizing hemangioma, one with a lymphoepithelioma of the tonsil with widespread metastasis, one with melanoma with numerous cutaneous metastases, and one with extensive mycosis fungoides were treated with P^{32} by Kenney.⁹ All but one of the patients with osteogenic sarcoma were still being treated at the time of the report, and no estimate of the end results of the therapy was possible. One patient who had refused amputation, and whose primary tumor had received a 9,100 “r” tumor dose of 1,000 kv. x-ray at 70 centimeters T.S.D., was given 20 millicuries of radioactive phosphorus over a period of three months. The extensive metastases continued to grow during the period of therapy with P^{32} . The patient with metastasizing hemangioma received a total dose of 150 microcuries per kilogram of body weight in eighteen days; this was divided into small daily doses. No regression was observed during life, nor were any changes that could be attributed to the P^{32} noted in the tumor tissue at autopsy twenty days after the commencement of therapy. The patient with metastases from a lymphoepithelioma of the tonsil was given 350 microcuries per kilogram of body weight in thirty-four days. No regression of the lesions was noted at any time. The patient with cutaneous metastases from a melanoma received 350 microcuries per kilogram of body weight in divided doses in forty days. The lesions increased steadily in size during and after the administration of P^{32} . The patient with mycosis fungoides was given 200 microcuries per kilogram of body weight in nineteen days. There was some regression of the skin lesions, but the individual left the hospital before the course of therapy was considered complete and he was not seen again.

Fitz-Hugh and Hodes¹⁰ administered P^{32} to two individuals with extensive gall bladder carcinoma and one with disseminated carcinoma of the breast. None of these patients was benefited by treatment with the isotope. The dosage employed was not stated.

B. *Analysis of the Results Obtained in Treating Three Patients With Ewing's Sarcoma, One With Malignant Melanoma, One With Anaplastic Carcinoma (Primary Site Undetermined), Two With Mycosis Fungoides, and One With Xanthomatosis.*—The diagnosis in all eight of these patients was confirmed by biopsy. Two of the patients had been treated previously with x-radiation. One individual (Patient 7, Table 19) had had mycosis fungoides for six years; at various times he had received fever therapy and several courses of x-ray, neither of which forms of treatment had had any significant effect on the course of the disease. Patient 3, a 17-year-old boy with Ewing's sarcoma, had just completed a course of x-ray therapy (2,400 roentgens to the primary tumor which arose from a rib on the right side); this considerably lessened the pain he was having in the right chest.

The total dosage of P^{32} administered to each of these individuals is recorded in Table 19. In two instances the dosage was very small. Patient 2, a girl, 9 years of age, with Ewing's tumor arising from the right ileum, was given 3.1 millicuries of P^{32} during eighteen days. Throughout this time she suffered from severe pain in the right hip and right leg; as this pain was becoming steadily worse, therapy with P^{32} was discontinued and x-radiation started. She was given 400 roentgens daily for twelve days, at the end of which time pain was somewhat less intense. She then returned to her home in another city, and no observations regarding the effect of the x-radiation on the size of the tumor are available. Patient 8, a male infant 12 months of age with xanthomatosis, weighed 15 pounds. He was given only 0.33 millicuries of radioactive phosphorus in three injections during a period of four days. Death oc-

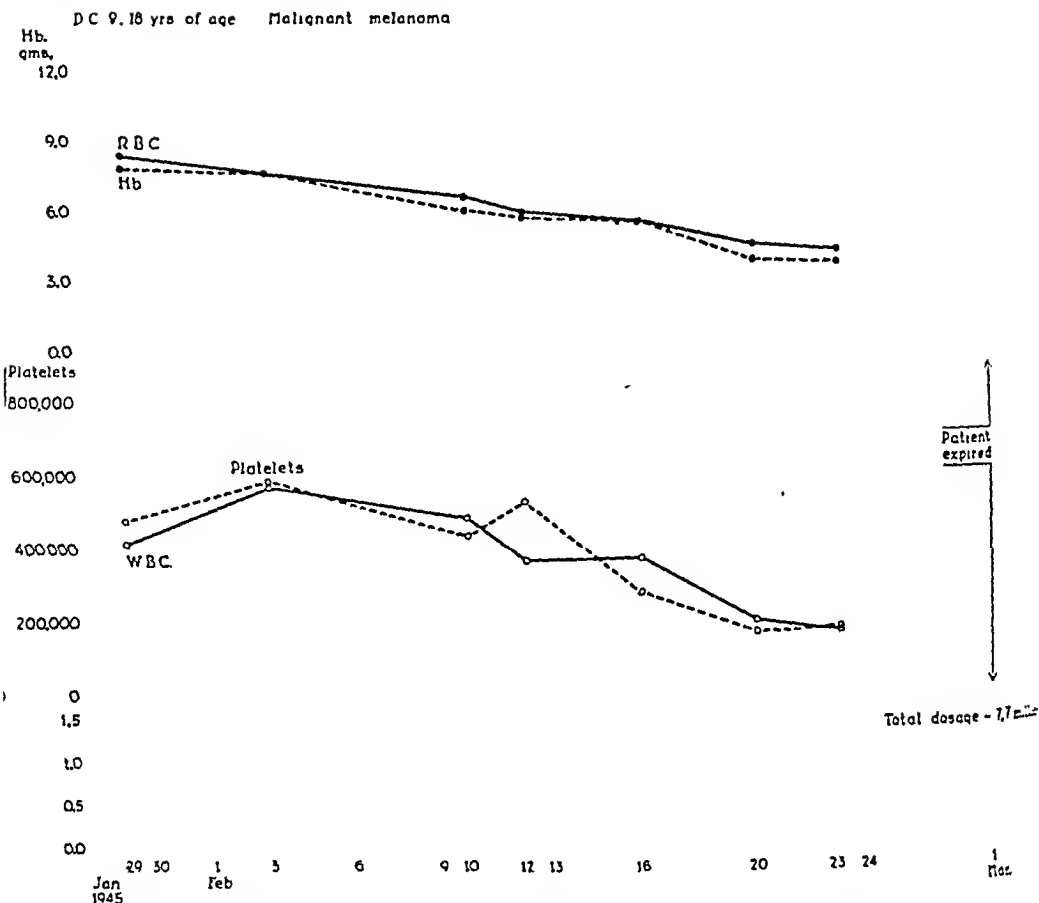


Fig. 18.—Malignant melanoma. Effect of P^{32} therapy on the blood.

Case Summary.—D. C., a young woman 18 years of age, had had numerous moles scattered over her body since childhood. In January, 1944, a mole which was increasing in size was removed from the left arm; at the same time large moles were removed from the back and from the left axilla. In August, 1944, she noted a lump in the left axilla; it enlarged rapidly. In November, 1944, this mass was biopsied and was found to be a malignant melanoma. A month later she was given x-ray therapy totaling 2,300 roentgens to the left axilla. About December 28 she developed severe low back pain, weakness, tinnitus, anorexia, weight loss, and fever.

Physical Examination: The temperature was 39° C. There was a striking pallor of the skin and mucous membranes. A stony hard mass measuring 5 by 5 cm. was present in the left axilla, and five stony hard, freely movable subcutaneous nodules were palpable in the abdominal wall. Breath sounds were distant, and the percussion note was hyporesonant bilaterally.

Laboratory Data: Blood counts (Jan. 29, 1945): leucocytes, 7,850 per cubic millimeter; erythrocytes, 2,840,000; hemoglobin, 8.0 Gm.; reticulocytes, 2.0 per cent; platelets, 486,000; differential, normal. Sternal bone marrow (Jan. 30, 1945): the marrow was very dense and hypercellular; 60 per cent of the cells in the marrow were malignant sarcoma cells. Urine was negative. Kahn reaction was negative. Serum proteins, normal.

(Continued on opposite page.)

curred nine days after treatment was started. It is recognized that in both of these patients the dosage employed was inadequate to permit evaluation of the treatment.

None of the patients considered in this section was benefited either subjectively or objectively by P^{32} therapy. In no instance did a palpable or roentgenologically demonstrable tumor mass decrease in size during or following administration of the isotope. Patient 3 (Ewing's tumor) was given 11.5 millicuries of P^{32} in fifty-three days; this dose was sufficient to produce profound anemia, leucopenia, and thrombocytopenia. In spite of the fact that therapy was pushed beyond the point of tolerance, several visible tumor masses arising from the skull bones increased at least 50 per cent in size during the course of isotope administration. Patient 4, a girl 18 years of age with melanosarcoma, was given 7.7 millicuries in twenty-six days, during which time she also developed anemia, leucopenia, and thrombocytopenia. Not only did the axillary and subcutaneous metastases present at the time P^{32} administration was started increase in size, but numerous new tumor masses appeared. Patient 5 had an anaplastic carcinoma involving the right nasopharynx and adjacent structures; there was marked exophthalmus on the right and extensive involvement of the frontal bone bilaterally. Even though 9.0 millicuries were administered in sixty-six days, all visible and roentgenologically demonstrable masses became larger; x-ray therapy (3,400 roentgens in thirteen days) was then administered, following which there was some decrease in size. A month later P^{32} therapy was resumed; this time he was given 3.9 millicuries within a period of six months. During this time, a stony hard mass appeared in the right side of the neck which increased in size until it measured 7 by 7 centimeters. A month after P^{32} was discontinued, he was given x-radiation, 300 roentgens daily for four days, to the lesion in the neck. One month later the mass in the neck had decreased 75 per cent in size. Both patients with mycosis fungoides (Patients 6 and 7) showed slow progression of their skin lesions while they were under treatment with P^{32} . Following discontinuance of P^{32} administration, Patient 6 was given weekly x-ray treatments by his dermatologist for one year. Approximately 1,600 roentgens were administered to most areas of the body; local areas of tumefaction received larger dosages than this. The local tumors decreased in size at first, but during the latter part of the year, the lesions again showed progression. The subsequent course of Patient 7 is not known.

Hematologic data and the case report for the patient with malignant melanoma (Patient 4) are given in Fig. 18.

Course: Following onset of therapy with P^{32} the pain in the back diminished. However, the patient's appetite steadily worsened, and weight loss and weakness rapidly progressed. High swinging fever persisted. By Feb. 15, 1945, she was semistuporous, and respirations were Cheyne-Stokes in character. During the first two weeks of therapy the mass in the left axilla decreased very slightly in size; thereafter both this mass and the subcutaneous nodules slowly enlarged. The patient died thirty-one days after the first dose of P^{32} had been given.

At post-mortem examination, metastases of malignant melanoma were found in the skin, the lungs, the liver, the spleen, the ovaries, the kidneys, the adrenals, the pancreas, the intestinal wall, the heart, the dura, the leptomeninges, and in lymph nodes throughout the body.

C. *Summary*.—Only a few patients with any of the diseases discussed in this section have been treated with P^{32} . However, the data on these individuals suggest that radioactive phosphorus, in the form in which it has been administered, is not a satisfactory therapeutic agent for the treatment of lympho-epithelioma, malignant melanoma, carcinoma of the gall bladder, carcinoma of the breast, Ewing's tumor, mycosis fungoides, or xanthomatosis.

XI. GENERAL SUMMARY AND CONCLUSIONS

Each section of this paper has been summarized independently, and only final conclusions will be recorded here.

Radioactive phosphorus emits beta-rays capable of producing a profound radiation effect on tissues. The therapeutic use of this substance has certain advantages compared with x-radiation: it is selectively concentrated in organs with a high phosphorus content such as bone and in tissues the cells of which are multiplying rapidly; it is easy to administer, and therapeutic does never give rise to radiation sickness. The half-life of P^{32} (14.3 days) permits steady radiation of tissues for several weeks, yet is short enough so that the destructive effect on tissues can be controlled.

The literature on radioactive phosphorus therapy has been analyzed, and the results obtained in the treatment of 155 patients at the Mallinckrodt Institute of Radiology have been presented. The available data appear to us to justify the following conclusions:

1. Radioactive phosphorus is probably the best therapeutic agent available at the present time for polycythemia vera. Complete hematologic and almost complete symptomatic remissions can be produced with P^{32} in the vast majority of patients, and remission from a single course of treatment may last for from six months to several years or longer. It is not possible at present to evaluate the effect of radioactive phosphorus therapy on the duration of life of these individuals.

2. Therapy with P^{32} has very little effect on the clinical course of patients with acute or subacute myelogenous leukemia but produces at least as complete clinical and hematologic remissions as x-radiation in the chronic form of the disease. Freedom from radiation sickness is a practical advantage which patients who have had previous x-ray therapy appreciate. The duration of life from the first symptom until death of the patients treated at the Mallinckrodt Institute of Radiology suggests that P^{32} therapy prolongs life to approximately the same extent as does x-ray therapy (about six months).

3. In the great majority of patients, the clinical course of acute lymphatic leukemia is not favorably influenced by P^{32} therapy. In a few patients clinical improvement has been observed, and one patient had almost complete hematologic and symptomatic remission of five and one-half months' duration. However, equally dramatic spontaneous remissions of acute lymphatic leukemia have been observed in our clinic and elsewhere. In the treatment of chronic lymphatic leukemia, P^{32} is probably as satisfactory as, but no better than, roentgen radiation.

4. Radioactive phosphorus is of no value in the treatment of monocytic leukemia.

5. Hodgkin's disease, lymphosarcoma, reticulum cell sarcoma, and multiple myeloma do not respond as favorably to P^{32} as they do to x-radiation.

6. From observations on the few patients who have been treated, there is no reason to believe that P^{32} is a satisfactory therapeutic agent for the treatment of lymphoepithelioma, malignant melanoma, carcinoma of the gall bladder, carcinoma of the breast. Ewing's tumor, mycosis fungoides, or xanthomatosis.

7. In patients with all the various types of diseases which have been treated, P^{32} has been shown to have a profound effect on the bone marrow, and severe leucopenia, thrombocytopenia, and anemia may occur as complications of the therapy. There is wide variation in the dosage of radioactive phosphorus required to produce these complications in different individuals. When more than one of the cellular elements of the marrow were depressed in the same individual, the cytologic changes in the peripheral blood usually occurred in the following order: the leucocyte level decreased first, the thrombocyte level second, and the erythrocyte level was affected last.

8. In patients with any type of chronic leukemia, roentgen radiation is more effective in some cases than P^{32} in bringing about a rapid reduction in the size of the spleen or lymph nodes. Therefore, x-ray should be employed whenever the prompt reduction of nodes or the spleen is necessary to relieve symptoms or remove pressure on some vital organ. X-ray may be used to supplement radioactive phosphorus therapy for this purpose.

9. Detailed suggestions as to methods of administering P^{32} and the proper dosage range for the treatment of patients with polycythemia vera, chronic myelogenous leukemia, and chronic lymphatic leukemia have been recorded. Therapy must be individualized to a high degree, and the final total dosage for any patient must be determined on the basis of repeated blood and bone marrow studies and clinical observations.

10. At present, several different methods are employed in different laboratories for the assay of the activity of solutions of radioactive phosphorus salts. These methods have been discussed by Dr. Martin D. Kamen in the Appendix. When the same sample of radioactive di-sodium acid phosphate solution was assayed in several laboratories, widely divergent results were obtained. This fact must be kept in mind in calculating dosage. An attempt is being made to correct this discrepancy.

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(APPENDIX FOLLOWS)

APPENDIX

APPENDIX TABLE 1. CLINICAL RESULTS

PATIENT	BEFORE TREATMENT WITH P ₃₂		AFTER P ₃₂
	SYMPTOMS	PHYSICAL SIGNS DUE TO POLYCYTHEMIA	
1	Fatigability, headaches, itching, dizzy spells, vertigo, numbness and aching of extremities (7 yr.)	Skin and mucous membranes dark red; cyanosis; conjunctivae red; spleen 6 cm. below costal margin	All symptoms persisted but became less severe and less frequent
2	Fatigability, headaches	Skin and mucous membranes red; spleen 3 cm. below costal margin	All symptoms improved greatly for 18 mo., then became worse, continued to have headaches
3	Itching and lacrimation (7 yr.), fatigability (1 yr.)	Spleen 12 cm. below costal margin	Each course of P ₃₂ therapy completely relieved all symptoms until R.B.C. count again rose
4	Fatigability, headaches, aching pains in legs, dizziness, dyspnea, depression, forgetfulness, nosebleeds, blurring of vision	Skin and mucous membranes dark red; petechiae and ecchymoses; conjunctivae red; apical systolic murmur; spleen 4 cm. below costal margin	All symptoms disappeared except headaches, dizziness, and dyspnea, which lessened in frequency and severity
5	Headaches, tinnitus, vertigo, fatigability (16 yr.); 6 epileptiform convulsions (11 yr.); itching, bloating, belching, numbness in extremities (3 yr.)	Skin and mucous membranes intensely red, cyanotic; fundi—venules distended; spleen 3 cm. below costal margin; liver 5 cm. below costal margin	No tinnitus, vertigo, fatigability or aching in extremities; occasional mild headaches, belching, and bloating; convulsions before
6	Headaches, numbness and tingling in hands and anorexia; 8 mo. prior to P ₃₂ therapy had sudden left hemiparesis which gradually cleared up	Skin and mucous membranes somewhat red; lips cyanotic; spleen 4 cm. below costal margin	No headaches or numbness and tingling in hands; appetite much better
7	Severe headaches with nausea and vomiting, transient episodes of unconsciousness, burning of eyes and lacrimation	None except spleen is 3 cm. below costal margin. B.P., 150/80	All symptoms completely relieved; still feels well 33 mo. after treatment
8	Numbness and pain in hands and feet, precordial pain, pain in both shoulders, fatigability, drowsiness	Skin and mucous membranes dark red, cyanotic; B.P., 185/115; heart enlarged; spleen 18 cm. below costal margin; liver 14 cm. below costal margin	Fatigability, drowsiness greatly improved; precordial pain and pain in shoulders and hands unimproved
9	Left hemiplegia (6 mo.), headaches, blurring of vision, fatigability, loss of memory, convulsion (4 mo. previously)	Skin and mucous membranes dark red, cyanotic; B.P., 150/120; apical systolic murmur; spleen 3 cm. below costal margin, left hemiplegia	Fatigability, headaches, vision and memory somewhat improved; no change in hemiplegia; continued to have convulsions
10	Severe headaches, fatigability, aching and tingling of extremities, lower abdominal pain	Spleen 4 cm. below costal margin	Headaches improved; fatigability no better; patient miserable because of pains "all over body," weakness, dyspnea, and palpitation
11	Headaches, dizzy spells, severe itching, substernal pain, aching in hips and thighs, moderate fatigability	Skin and mucous membranes red; ecchymoses on thighs; B.P., 155/100; spleen tip palpable just at costal margin	Itching and substernal pain disappeared entirely; other symptoms lessened in severity
12	Sensation as though head were twice normal size, headaches, dizziness, nervousness, blurring of vision	Skin and mucous membranes brilliantly red; nails and lips cyanotic; engorgement of venules (fundi); B.P. 150/120; spleen 12 cm. below costal margin; liver 4 cm. below costal margin	All symptoms completely relieved except for nervousness and occasional slight dizzy spells

THIRTY PATIENTS WITH POLYCYTHEMIA

PHYSICAL SIGNS	REMARKS
abnormal findings; spleen not palpable	Course complicated by marked nervousness, severe menopausal symptoms, and marital strife
abnormal findings except barely palpable spleen for 15 mo.; many findings thereafter	Two years after onset of therapy, patient developed progressive leucocytosis, anemia, thrombocytopenia; died with "myelogenous leukemia"
spleen 1-4 cm. below costal margin	Phlebotomies (12 liters in 4 yr.) prior to P ³² therapy only partially relieved symptoms
for normal; no petechiae or ecchymoses; conjunctivae clear; no murmurs; spleen not palpable	Patient 60 yr. old; has had hypertension (160-180/100-110) for many years; fundi show sclerotic changes, and EKG showed "myocardial damage"
for normal; no distention of venules (fundus); spleen barely palpable on deep inspiration; liver not palpable	Patient 42 yr. old; electroencephlogram did not show changes suggestive of epilepsy; much greater relief from P ³² than from phenylhydrazine taken previously
for normal; spleen not palpable	Patient also had fine tremor (? postencephalitic) and hypogonadism; left town 6 mo. after treatment; not seen thereafter
abnormal findings; spleen not palpable; B.P., 125-135/70-80	Treated for 4 yr. prior to P ³² therapy by repeated venesection (about 500 c.c. every eight weeks) with partial relief of symptoms
for pale; B.P., 150-160/80-110; heart unchanged; spleen 5 cm. below costal margin; liver 10 cm. below costal margin	EKG showed right bundle branch block; x-ray of spine showed marked hypertrophic spurring of cervical vertebrae
for normal; B.P., 110-120/70-95; no murmurs; spleen not palpable; hemiplegia unchanged	Neurologic signs suggested thrombosis with encephalomalacia involving the right pyramidal system as cause of the hemiplegia
edge of spleen just felt at costal margin on inspiration	Previously treated with Fowler's solution, phenylhydrazine, and phlebotomies with indifferent results; diagnosed by psychiatrist as having severe psychoneurosis
abnormal findings except slight hyperextension; B.P., 145-160/80-100	During last year patient has complained of numbness of feet and pain and stiffness in hands; x-rays negative
for normal; no cyanosis or engorgement of venules; B.P., 140/100; spleen 7 cm. below costal margin; liver not palpable	Patient previously treated with phenylhydrazine; symptomatic relief not as good as following P ³² ; delivered normal baby nine months after last injection of P ³²

APPENDIX TABLE 1. CLINICAL RESULTS

PATIENT	BEFORE TREATMENT WITH P32		AFTER TREATMENT
	SYMPTOMS	PHYSICAL SIGNS DUE TO POLYCYTHEMIA	
3	Redness of eyes and lacerimation, blurring of vision, itching, soreness and bleeding of gums	Skin slightly redder than normal; spleen 6 cm. below costal margin; liver 3 cm. below costal margin	All original symptoms relieved except blurring of vision (glasses checked and found to be correct)
4	"Dazed feeling," headaches and shooting pains in head, aching in extremities, itching of skin, fatigability, palpitation; blind in left eye for 2 yr.	Skin and mucous membranes bright red; slight cyanosis; left optic atrophy; moderate pulmonary emphysema; B.P., 150/90; spleen 9 cm. below costal margin; liver 6 cm. below costal margin	No symptoms except very mild headaches and occasional dizziness; mild itching now confined to face only; blindness of left eye unchanged
15	Severe constant burning of tongue and bitter taste in mouth, moderate itching of skin, fatigability	Skin and mucous membranes bright red; "erythema multiforme" on trunk; conjunctivae red; spleen edge felt just at costal margin; liver not palpable; B.P., 150/80	Itching of skin and fatigability disappeared entirely; burning of tongue and bitter taste bothers her only intermittently
16	Constant headaches, weakness and fatigue, deep pain in both legs, severe itching, pain and burning in eyes, clumsiness of left arm and leg	Skin and mucous membranes brilliant red; cyanosis; blotchy erythema on chest; conjunctivae red; spleen tip palpable just at costal margin; liver not palpable; B.P., 150/90	Patient now has only rare headaches and occasional aching in thighs; all other symptoms completely relieved
17	Severe pain in both legs, burning of eyes and lacerimation, epistaxis, nervousness, excessive perspiration	Skin and mucous membranes moderately red; slightly cyanotic; conjunctivae red; venules distended (fundi); spleen 11 cm. below costal margin; feet wet and cold	All symptoms completely relieved
18	Fatigability, headaches, dizzy spells, itching, aching and numbness of extremities, blurring of vision	Skin and mucous membranes slightly redder than normal; spleen palpable right at the costal margin	Patient still has occasional headaches, rare dizzy spells and pains in legs
19	Nervousness for many years, dizziness and nausea (1 yr.), severe chorea and inability to talk coherently (1 mo.)	Face red; heart moderately enlarged; apical systolic murmur; spleen tip palpable on inspiration	Patient still somewhat nervous; all other symptoms completely relieved
20	Languor, drowsiness, difficulty concentrating, lack of interest in his work, blurring of eyes and of vision	Quite thin; mucous membranes bright red; marked tortuosity and distention of vessels in fundi; spleen palpable right at costal margin	All symptoms completely relieved for 6 mo.; drowsiness and lack of interest then returned (blood still normal)
21	Partial right hemiplegia (6 wk.), incoherent speech, dyspnea, blurring of vision, severe depression	Skin and mucous membranes slightly ruddy; right arm paralyzed; right facial paralysis; speech incoherent; pulmonary emphysema; spleen felt at costal margin	Dyspnea, blurring of vision, depression have completely cleared up; speech much improved; right arm still paralyzed
22	Severe headaches, marked weakness and fatigability	Skin and mucous membranes bright red; cyanosis; B.P., 170/115; spleen not palpable	All symptoms completely relieved

-TY PATIENTS WITH POLYCYTHEMIA—CONT'D

APY PHYSICAL SIGNS	REMARKS
abnormal findings; liver and spleen not palpable	One month after last injection of P^{32} patient stopped menstruating; ever since then she has had hot flashes and nervousness; venesection (for 4 mo. prior to P^{32}) relieved most of symptoms
r normal; optic atrophy and pulmonary nphysema as before; B.P., 130-150/80-90; spleen not palpable; liver 2 cm. below costal margin	Patient previously treated by phlebotomies with partial relief of symptoms; patient had been unable to work for 10 yr. prior to P^{32} therapy; now works 11-12 hours a day on his farm
abnormal findings except B.P., 145-50/80; spleen not palpable	For years, patient had made it a practice to rub red pepper on tongue to relieve the bitter taste; sometimes did this several times a day
abnormal findings except B.P., 140-50/80-90	8 mo. before P^{32} therapy was started he had a sudden paralysis of left arm and leg; this gradually improved during period of several months, leaving only slight clumsiness
abnormal findings; spleen not palpable	Patient previously treated with phenylhydrazine with very poor results; then controlled by repeated phlebotomies which gave temporary relief from all symptoms except severe pain in legs
abnormal findings; spleen not palpable	1 phlebotomy (250 c.c.) performed 1 mo. after last injection of P^{32} (first course); 7 pains in legs due to varicosities
abnormal findings except an apical systolic murmur	Patient's mother had polycythemia; 6 wk. prior to P^{32} therapy patient had 4 x-ray treatments; 3 wk. prior to P^{32} therapy 5 phlebotomies (totaling 2300 c.c.) performed; chorea improved before P^{32} given
ined 10 pounds; color normal; spleen not palpable	Previously treated with phenylhydrazine and venesections with little relief of symptoms; recent return of symptoms due to severe depression brought on by fiancée breaking engagement
or normal; speech still difficult to understand but greatly improved; spleen no longer palpable; other physical signs unchanged	Patient treated with phenylhydrazine for several weeks and 2 phlebotomies performed before patient came to St. Louis for treatment; 3 additional phlebotomies performed just prior to P^{32} therapy
or normal; B.P., 180-220/120-135	2 phlebotomies (450 c.c. each) performed, 1 on day P^{32} given and other 2 days later; EKG showed evidence of severe myocardial damage

APPENDIX TABLE 1. CLINICAL RESULTS

PATIENT	BEFORE TREATMENT WITH P32		AFTER
	SYMPTOMS	PHYSICAL SIGNS DUE TO POLYCYTHEMIA	
23	Severe headaches, dizziness, itching, dyspnea, palpitation, attacks of substernal pain associated with breathlessness, fatigability	Skin and mucous membranes bright red; cyanosis; conjunctivae red; B.P., 150/100; heart moderately enlarged; apical systolic murmur; expiratory wheezes throughout both lungs; spleen 5 cm. below costal margin; liver 10 cm. below costal margin	All previous symptoms cleared entirely except itching which decreased greatly in intensity; however, patient now has occasional uncontrollable "erythematous spells" and loss of libido
24	Mild headaches, dizzy spells, fatigability, aching in thighs and legs, brief episodes of inability to move legs, frequent bruises, itching	Skin and mucous membranes bright red; conjunctivae red; venules greatly distended (fundi); B.P., 155/100; frequent premature systoles; spleen tip felt right at costal margin.	All symptoms greatly improved; only significant complaints lack of pep and mild itching
25	Mild headaches, dizzy spells, aching pains in extremities, drowsiness, itching, blurring of vision, epigastric pain and vomiting	Skin and mucous membranes red; cyanosis; conjunctivae red; vessels of fundi sclerotic; pulmonary emphysema; spleen tip felt 2 cm. below costal margin	Dizziness, aching in extremities, drowsiness, and blurring of vision cleared up entirely; headaches now mild and infrequent; itching and ulcerous lesions unimproved
26	Drowsiness, uncontrollable yawning, mild headaches, dizzy spells, itching, pains in back and thighs, numbness of hands and feet, frequent bruises, polyuria, nervousness, hot flashes	Skin and mucous membranes bright red; cyanosis; distention of venules (fundi); spleen 13 cm. below costal margin; liver 6 cm. below costal margin	Patient still bothered by drowsiness, an occasional spell, bruises, cramps in legs, and hot flashes; all other symptoms have cleared up generally, greatly improved
27	Headaches, weakness and fatigability, dizziness, itching, tinnitus, repeated "attacks" characterized by severe vertigo, blurring of vision and rigor	Asthenic; malnourished; distention of venules (fundi); spleen 2 cm. below costal margin	All symptoms greatly improved but still has occasional headache; feels much stronger; no "attacks"
28	Severe headaches, profound fatigability, dyspnea on exertion, swelling of ankles	Skin and mucous membranes bright red; pronounced cyanosis; conjunctivae red; venules of fundi distended; spleen 2 cm. below costal margin; liver 3 cm. below costal margin; B.P., 150/100	All previous symptoms now completely relieved; now has no edema of right leg
29	Headaches, pain in legs, itching, blurring of vision, repeated "heart attacks" characterized by pain in chest and epigastrium, palpitation, dyspnea	Skin and mucous membranes bright red; cyanosis; heart and lungs normal; B.P., 130/80; spleen not palpable; clubbing of fingers	All symptoms completely relieved except for an occasional headache and dizzy spell
30	Fatigability, dyspnea, headaches, dizzy spells, aching in extremities, itching, smarting of eyes and lacrimation, indigestion	Spleen 8 cm. below costal margin; no other abnormal findings	All symptoms relieved

FIFTY PATIENTS WITH POLYCYTHEMIA—CONT'D

THERAPY	REMARKS
PHYSICAL SIGNS	
<p>Color normal; B.P., 120-135/85-100; auricular fibrillation; lungs clear but breath sounds distant; spleen not palpable; liver 8 cm. below costal margin</p>	<p>During 3 wk. preceding first injection of P^{32} 8 phlebotomies done (3,900 c.c. blood withdrawn); during next 2 mo. 2 more phlebotomies done (500 c.c. each); EKG showed only flat T waves in first three leads</p>
<p>abnormal findings except auricular fibrillation; spleen barely palpable on deep inspiration</p>	
<p>Color normal; spleen not palpable; other findings unchanged</p>	<p>Gastrointestinal x-rays showed pyloric obstruction</p>
<p>Color normal; spleen tip palpable just at costal margin</p>	<p>8 yr. prior to first therapy with P^{32} patient had epigastric pain relieved by food; four years later had hematemesis; x-rays at this time showed duodenal ulcer</p>
<p>abnormal findings</p>	<p>Previously treated by phlebotomies with partial relief of symptoms</p>
<p>abnormal findings except pitting edema of right leg</p>	<p>Previously treated by phlebotomies with great improvement but not as good as following P^{32}; 2 yr. ago patient had typical peptic ulcer symptoms which cleared up on diet and alkalies; 4 phlebotomies (500 c.c. each) done during first 2 wk. of P^{32} therapy; developed thrombosis of deep veins of right leg 4 days after P^{32} therapy</p>
<p>Color and mucous membranes still redder than normal; cyanosis less marked; clubbing of fingers as before</p>	<p>Previously treated with phenylhydrazine and phlebotomies without significant relief of symptoms; patient apparently had a coronary occlusion 4 yr. previously, confirmed by EKG; patient has only been followed for 3 mo. since initial injection of P^{32} was given, and blood counts have not yet returned to normal</p>
<p>Spleen smaller (actual measurements not taken); no other abnormal findings</p>	<p>Patient was treated for 1 yr. by phlebotomies (500 c.c. withdrawn every 10-14 days); last phlebotomy 10 days before P^{32} injections; each phlebotomy relieved symptoms for about 1 wk.</p>

APPENDIX TABLE 2. MYELOGENOUS LEUKEMIA—SYMPTOMATIC RESPONSE TO THERAPY

PATIENT	WEAKNESS AND FATIGABILITY	POUNDING IN HEAD	DIZZINESS	DYSPNEA ON EXERTION	PAIN IN EXTREMITIES	PALPITATION	BLEEDING—VAGINA, NOSE OR GUMS	SORENESS OF GUMS	SPONTANEOUS BRUISES	SEMI-CONSCIOUSNESS OR STUPOR	NIGHT SWEATS	CHILLS AND FEVERISHNESS	HEADACHES	TINNITUS	NERVOUSNESS	DIARRHEA	BLOATING, DYSPEPSIA	ANOREXIA	NAUSEA AND/OR VOMITING	ABDOMINAL PAIN	SWELLING OF LEGS, GENITALS	WEIGHT	COUGH	VISION
1	++ ++ ++ ++ +	0 0 +	0 0 +	++ ++	0 + 0			++ +	+ 0						+++ ++ +			0 0 +	0 0 +					
2	++ ++ ++ ++ +	0 0 +			++ +										+++ ++ +									
3	++ ++ ++ ++ +	0 0 +		0 0 +									0 0 +		+++ ++ +									
4	++ ++ ++ ++ +	0 0 +					+					0 0 +			+++ ++ +					+				
5	++ ++ ++ ++ +	0 0 +				0 0 +	+						0 0 +	0 0 +	+++ ++ +					+				
6	++ ++ ++ ++ +	0 0 +	0 0 +	++ ++		0 0 +	+						0 0 +	0 0 +	+++ ++ +					+				
7	0 0 +	0 0 +					+						0 0 +	0 0 +	+++ ++ +					+		63+		
8	++ ++ ++ ++ +	0 0 +			0 0 +							0 0 +			+++ ++ +					+				
9	++ ++ ++ ++ +	0 0 +			0 0 +							0 0 +			+++ ++ +					+				

[illegible]

APPENDIX TABLE 2. MYELOGENOUS LEUKEMIA—SYMPTOMATIC RESPONSE TO THERAPY—CONT'D

PATIENT	WEAKNESS AND FATIGABILITY	FOUNDING IN HEAD	DIZZINESS	DYSPNEA ON EXERTION	PAIN IN EXTREMITIES	PALPITATION	BLEEDING—VAGINA, NOSE OR GUMS	SORENESS OF GUMS	SPONTANEOUS BRUISES	SEMI-CONSCIOUSNESS OR STUPOR	NIGHT SWEATS	CHILLS AND FEVERISHNESS	HEADACHES	TINNITUS	NERVOUSNESS	DIARRHEA	BLOATING, DYSPEPSIA	ANOREXIA	NAUSEA AND/OR VOMITING	ABDOMINAL PAIN	SWELLING OF LEGS, GENITALS	WEIGHT	COUGH	BURNING OF VISION	
23	++ 0			++ ++		+	0 0										++ ++	++ ++	++ ++	+	+	0 +	+16	++ ++	
24	++ ++			++ ++		+	0					++ ++			+	+	++ ++	++ ++		+	+	+	+5	++ ++	
25	+++ +++ +++		+++ +++ +++	+++ +++ ++		+						+++ ++			+	+	+++ ++	++ ++	++ ++	+++ ++	+++ ++		No chg.	++ ++	
Expired on third day of treatment																									
26	0																					+10			
27	0																0	0					+5		
28	++ ++			+													+	++ ++	++ ++			+	No chg.		
Expired on fifth day of treatment																									
29	++ ++				+		+								++ ++					+	+		+25		
30	++ ++				0		+								++ ++		+	+			0		+33		
31	++ ++		++ ++	++ ++		++ ++	+										++ ++	++ ++	++ ++			+	+13		

APPENDIX TABLE 3. MYELOGENOUS LEUKEMIA—CHANGE IN PHYSICAL SIGNS FOLLOWING P₃₂ THERAPY

PA- TIENT	FEVER (1)	TACHY- CARDIA (2)	PETECIAE OR ECCHYMOSES (3)		BLEEDING FROM NOSE OR GUMS (4)	ENLARGED LYMPH NODES (5)	SYSTOLIC CARDIAC MURMUR (6)	SPLENO- MEGALY (7)	HEPATO- MEGALY (7)	EDEMA OF EXTREMI- TIES	SKIN ERUPTION (OTHER THAN PETE- CHIAE)	ASCITES	OTHER PHYSICAL SIGNS
			SKIN	MUCOUS MEMBRANES, FUNDI, OR CONJUNCTIVAE									
1	0 0 ++	0 0 +	0 0 ++	0 0 +	0 0 +	+	0 0 0	4-8 3-6 5-11	X-2 X-X 3-9	0 0 0	0 0 0		
2	0 0 +	0 0 +	0 0 +	0 0 +	0 0 +	0 0 ++	0 0 0	X-X X-X 3-7	X-X X-X X-X	0 0 0	++ ++ +		
3	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	X-2 X-X X(-2)	X-2 X-X X-X	0 0 0	0 0 0		B.P., 155/100
4	+	0 0 0	0 0 0	0 0 0	0 0 +	+++ +++ +++	0 0 0	4-9 4-9 1-9	X-X X-X X-X	0 0 ++	0 0 0	0 0 ++	
5	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	X-X X-X X-X	X-X X-X X-X	0 0 0	0 0 0	0 0 0	B.P., 185/110
6	++ ++ ++	++ ++ ++	0 0 0	0 0 0	0 0 0	++ ++ ++	+	16-21 6-12 18-23	2-6 0-0 2-6	++ ++ +	0 0 +	0 0 0	
7	0 0 0	0 0 0	++ 0 0	0 0 0	0 0 0	0 0 0	+	9-11 X-X X-X	2-7 X-X X-X	0 0 0	0 0 0	0 0 0	
8	+	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	+	4-10 1-4 2-6	X-X X-X X-X	++ ++ +	0 0 0	++ 0 0	
9	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0	6-10 X-1 X-1	1-4 X-X X-X	0 0 0	0 0 0	0 0 0	B.P., 150/100
10	+	0 0 0	0 0 0	0 0 0	0 0 0	+	0	5-10 1-1 3-7	4-7 1-3 2-6	0 0 0	0 0 0	0 0 0	

[illegible]

APPENDIX TABLE 3. MYELOGENOUS LEUKEMIA—CHANGE IN PHYSICAL SIGNS FOLLOWING P32 THERAPY—CONT'D

PA- TIENT	TACHY- CARDIA (1)	TACHY- CARDIA (2)	PETECHIAE OR ECCHYMOSES (3)		BLEEDING FROM NOSE OR GUMS (4)	ENLARGED LYMPH NODES (5)	SYSTOLIC CARDIAC MURMUR (6)	SPLENO- MEGALY (7)	HEPATO- MEGALY (7)	EDEMA OF EXTREM- ITIES	SKIN ERUPTION (OTHER THAN PETE- CHIAE)	ASCITES	OTHER PHYSICAL SIGNS
			SKIN	MUCOUS MEMBRANES, FUNDI, OR CON- JUNCTIVAE									
24	0	+	0	0	0	+	+	3-9	5-8	0	0	0	
	0	0	0	0	0	0	0	2-4	3-5	0	0	0	
	+++	+++	0	0	0	+++	+	8-10	4-6	0	0	0	
25	+++	+++	0	0	0	0	+++	11-16	14-20	0	0	0	
	+++	+++	0	0	0	0	+++	11-16	14-20	0	0	0	
						<i>Expired on third day of treatment</i>							
26	+	++	0	0	0	+	0	x-x	x-x	0	0	0	
	0	+	0	0	0	0	0	x-x	x-x	0	0	0	
	0	0	0	0	0	0	0	x-x	x-x	0	0	0	
27	0	0	+	0	0	+++	0	6-11	x-x	0	0	0	
	0	0	0	0	0	+	0	5-10	x-x	0	0	0	
	0	0	0	0	0	+	+	10-24	3-6	0	0	0	
28	0	0	0	0	0	+	++	9-12	4-6	0	0	0	
	+	+	0	0	0	+	++	9-12	4-6	0	0	0	
						<i>Expired on fifth day of treatment</i>							
29	+	0	0	0	0	+	+	9-15	5-5	0	0	0	
	0	0	0	0	0	0	0	x-x	x-x	0	0	0	
	0	0	0	0	0	0	0	x-x	x-x	0	0	0	
30	0	+	0	0	0	0	++	11-19	8-11	0	0	0	
	0	0	0	0	0	0	+	4-7	3-6	0	0	0	
	0	0	0	0	0	0	0	4-8	5-8	0	0	0	
31	0	+	0	0	0	0	+	1-x	0-x	0	0	0	
	0	0	0	0	0	0	0	x-x	x-x	0	0	0	
	0	0	0	0	0	0	0	(-2)-x	x-(0)	0	0	0	
32	0	++	0	0	0	+++	0	8-13	5-9	0	0	0	
	0	0	0	0	0	++	0	(-2)-x	x-x	0	0	0	

	0	1	0	0	0	0	0	+	0	+	0	15-23	3-7	0	0
.33	++	++	0	0	0	0	0	+	0	+	0	11-19	3-5	0	0
	++	++	0	0	0	0	0	+	0	+	0	18-23	3-5	0	0
.31	+	++	0	0	0	0	+	1+	0	0	0	12-18	8-10	0	0
	+	++	0	0	0	0	0	0	0	0	0	x-x	x-x	0	0
	0	+	0	0	0	0	0	0	0	0	0	x-x	x-x	0	0
.35	0	+	0	0	0	0	0	++	+	+	0	7-10	6-7	0	0
	0	0	0	0	0	0	0	0	0	0	0	x-x	x-2	0	0
	0	0	0	0	0	0	0	0	0	0	0	x-x	x-2	0	0
.36	0	+	0	0	0	0	0	+	0	+	0	10-16	x-x	0	0
	0	0	0	0	0	0	0	+	0	+	0	(0)	x-x	0	0
	0	0	0	0	0	0	0	+	0	+	0	(0)	x-x	0	0
.37	0	+	0	0	0	0	0	++	0	++	0	10-19	3-6	0	0
	0	0	0	0	0	0	0	+	0	+	0	1-10	3-5	0	0
	0	0	0	0	0	0	0	+	0	+	0	1-10	3-5	0	0
.38	0	0	0	0	0	0	+	++	+	++	+	7-9	3-5	0	0
	0	0	0	0	0	0	+	++	+	++	+	(-2)-x	3-1	0	0
	0	0	0	0	0	0	0	++	+	++	0	(-2)-x	3-5	0	0
.39	0	0	0	0	0	0	0	+	0	+	0	7-13	2-6	0	0
	0	0	0	0	0	0	0	0	0	0	0	1-8	x-x	0	0
	0	0	0	0	0	0	0	0	0	0	0	1-8	x-x	0	0

The upper row of figures in each column represents the physical signs at the time the therapy was started; in the second row is recorded the maximum improvement in each physical sign during the first year of therapy; in the third row are recorded the physical signs at the end of the first year of treatment or when last seen (if the patient has been followed less than one year) or at death (if the patient died in less than one year).

(1) *F*over: Less than 37.5° C. = 0; 37.5° to 38.5° C. = +; 38.5° to 39.5° C. = ++; 39.5° to 40.5° C. = +++ (average including daily temperature).

(2) Tachycardia: Less than 80 = 0; 80 to 100 = +; 100 to 120 = ++; 120 to 140 = +++ (average maximum daily pulse).

(3) Graded 0 to +1+ or rough estimate of number and size of patches present.

(3) $\text{Crimed} = 0$ is iff absent.
(1) $\text{F} = \text{Present}$; 0 = absent.

(5) 0 = no palpably enlarged nodes; shotty nodes up to 0.5 cm. in diameter = +; nodes up to 1 cm. in diameter = ++; nodes 1 to 3 cm. in diameter = +++; nodes more than 3 cm. in diameter = ++++.

unheard = +††, notes more than 2 cm in diameter = +†††.

(7) Figures give actual measurement of spleen or liver edge below the costal margin in the anterior axillary line and in mid-clavicular line.

[illegible]

APPENDIX TABLE 4. LYMPHATIC LEUKEMIA—SYMPTOMATIC RESPONSE TO THERAPY—CONT'D

PATIENT	WEAK- NESS AND FATIGA- BILITY	FOUND- ING IN HEAD	DIZZI- NESS	DYSP- NEA ON EXER- TION	PALPI- TA- TION	HEAD- ACHES	SPONTA- NEOUS BRUI- ING	BLEED- ING FROM VAGINA, NOSE OR GUMS	BLOAT- ING, DYS- PEP- SIA	NAUSEA AND/OR VOMIT- ING	ANO- REXIA	AB- DOM- INAL PAIN	COUGH IN EX- TREM- ITIES	PAIN IN EX- TREM- ITIES	SORE- NESS OF GUMS	EXCES- SIVE SWEAT- ING	SWELL- OF LEGS OR GEN- ITALS	WEIGHT
25	++ + +			+++ ++ 0	++ 0 0									0 + +				- 1
26	++ ++ ++++		+ ++		++ ++ ++			+ ++ +	+ ++ +		+ ++ +							
27	+++ ++ +++		++ ++ ++	+ 0 +++	++ 0 ++	++ 0 ++		+ 0 +	+++ ++ +		+++ ++ +		+++ ++ +++					- 7
28	++ 0 0																	
29	+++ ++ +						++ 0 0											
30	+++ ++ ++											++ 0 0						+ 1

The upper row of figures in each column represents the symptoms present at the time the therapy was started; in the second row is recorded the maximum improvement observed in each symptom during the first year of therapy; in the third row are recorded the symptoms at the end of the first year of treatment or when last seen (if the patient has been followed for less than one year), or just prior to death (if the patient died in less than one year).

Zero indicates that the symptom was not present. If a symptom was present, the severity of the symptom is recorded as follows: Mild = +; moderate = ++; severe = +++; very severe = ++++.

APPENDIX TABLE 5. LYMPHATIC LEUKEMIA—CHANGES IN PHYSICAL SIGNS FOLLOWING P³² THERAPY

APPENDIX TABLE 3. LYMPHATIC ENLARGEMENT.

PATIENT	FEVER (1)	TACHY- CARDIA (2)	PETECHIAE OR ECCHY- MOSES (3)		ENLARGED LYMPH NODES (4)	SYSTOLIC CARDIAC MURMUR (5)	SPLENO- MEGALY (6)	HEPATO- MEGALY (6)	EDEMA OF EXTREM- ITIES	SKIN ERUPTION (OTHER THAN PETECHIAE)	ASCITES	OTHER PHYSICAL SIGNS
			SKIN	MUCOUS MEM- BRANES, FUNDI OR CONJUNCTIVAE								
1	++	+++	+++	++	+++		3-8 0-0 0-0		++		+	Enlargement of tonsils +++ Enlargement of tonsils ++ Enlargement of tonsils +++ B.P., 220/100
2		+			+	++	0-4 0-3 0-2	0-2 0-0 0-0				B.P., 230/125
3					+++		x-x x-x x-x	x-x x-x x-x				B.P., 150/70
4			+++ +++ +++	++ ++ ++	+	++	0-1 0-1 0-1	1-2 2-4 2-4				
5			+++	++	+++		x-0 x-x x-x	x-x x-x x-x				
6	++				++		3-8 2-5 3-8	x-x x-x x-x				
7					+++		4-8 2-5 2-5	2-5 1-3 1-3			++	
8				+	+++		4-9 2-5 5-11	1-4 x-x 2-6				
9					+		x-x * *	x-x * *				
10					+++		3-8 x-x x-x	2-5 1-3 x-x				B.P., 178/116

22			+	+	+	+	+	+	15-17 13-18 15-18	6-9 5-8 7-10						Enlargement of tonsils ++
23		+		+	+	+	+	+	6-12 x-x x-x	6-10 x-x x-x	+	+				Enlargement of tonsils +
24									x-x x-x x-x	x-x x-x x-x						
25		+	+	+	+	+	+	+	8-14 1-7 4-7	4-9 x-x x-x						
26	0 0 +	0 0 +	0 0 +	0 0 +	0 0 +	0 0 +	0 0 +	0 0 +	x-x x-x 1-x	x-x x-x x-1		+				
27				+	+	+	+	+	8-15 4-8 6-10	1-9 1-9 7-10						Rules + Rules 0 Rules ++
28			+	+	+	+	+	+	x-x x-x x-x	x-x x-x x-x						B.P., 175/100 B.P., 175/100 B.P., 175/100
29		+	+	+	+	+	+	+	3-x 0-x 0-x	1-1 1-1 2-6						
30									8-15 7-13 7-13	1-1 1-1 1-1						

In the upper row of figures in each column are recorded the physical signs at the time p_{22} therapy was started; in the second row is recorded the maximum improvement in each physical sign during the first year of p_{22} therapy; in the third row are recorded the physical signs at the end of the first year of treatment or when last seen (if the patient has been followed less than one year) or at death (if the patient died in less than one year).

(1) Fever: Less than 37.5° C. = 0; 37.5° to 38.5° C. = +; 38.5° to 39.5° C. = ++; 39.5° to 40.5° C. = +++ above 40.5° C. = ++++ (average maximum daily temperature).

(2) Twelve-year: Less than 80 = 0; 80 to 100 = +; 100 to 120 = ++; 120 to 140 = +++; above 140 = ++++ (average maximum daily pulse); 100 to 120 or more beats per minute or more of petechiae present.

(3) Graded 0 to +111 on rough estimate of number and size of petechiae present.

(3) Graded 0 to +++++ on rough estimate of number and size of pores present.

(5) Very loud murmur = +++; loud murmur = ++; faint murmur = +; no murmur = 0.

(6) Figures give actual measurement of spleen or liver edge below the costal margin in the anterior axillary line, and in mid-clavicular line.

X-ray therapy administered to spleen.

50% X-ray therapy administered to inguinal nodes.

APPENDIX

REMARKS ON THE ASSAY OF RADIOACTIVE PHOSPHORUS

MARTIN D. KAMEN, PH.D.

The activity of any radioactive substance is referred to an absolute standard, the "curie." Any substance emitting the same number of primary particles per second as does one gram of pure elementary radium is said to possess an activity of one curie. The most recent value for the number of disintegrations experienced by one gram of radium per second is 3.47×10^{10} . The assay of a radiophosphorus preparation then involves the determination of the number of disintegrations P^{32} undergoes per second referred to this unit. The P^{32} radiation, unlike that of radium which emits alpha and gamma rays, consists almost wholly of beta particles exhibiting the usual continuous beta spectrum and possessing a maximum energy of 1.69 millions of electron volts (M.E.V.). Detection of beta-rays involves an experimental setup radically different from that required in work with alpha and gamma rays. It is apparent, then, that a beta-ray standard is needed with which P^{32} samples can be compared and which, in turn, can be calibrated properly with respect to the curie standard.

It is not a simple matter to translate into curies the response of the various measuring instruments available, since the beta-rays produce an effect which is dependent on the geometry employed and the amount of absorbing matter through which they pass. In addition, the energy with which the beta-rays enter the sensitive volume of the detector determines the efficiency with which they are detected. In this brief note, only methods widely practiced at present for assaying radiophosphorus will be described. It is obvious that development of newer types of detectors expedited by war research should greatly modify the procedures employed in determinations of P^{32} dosage in future work.

Of the various detectors possible, two main types are employed: namely, electroscopes and Geiger counters. The voluminous literature on these instruments which has been made available in recent years renders unnecessary any discussion of them here or the principles underlying their use. The main type of electroscope used is the Lauritsen.* This instrument is a refinement of the familiar gold-leaf electroscope, the main improvement being the substitution of a gold-covered single fiber of quartz for the gold-leaf. The electrostatic capacity is about 0.2 centimeters and the charge sensitivity about 1 scale division for every 10^6 ion pairs. The motion of the quartz fiber is observed through a telescope ocular. In most cases, the instrument is supplied with a rather thick aluminum wall. To increase sensitivity to beta-rays, a portion of this wall is cut away and a thin aluminum foil ($\sim \frac{1}{2}$ mil thick) substituted.

A variety of Geiger counters is available, but since the samples are most conveniently mounted in dishes or flat planchettes, the open end "bell-jar" type

*See Lauritsen, C. C. and Lauritsen, T.: *Rev. Scient. Instruments* 8: 438, 1937.

of counter is most often employed. The Geiger counter is many orders of magnitude more sensitive than the Lauritsen electroscope but more complicated to maintain and operate. With either kind of detector two methods are available which one may label "absolute" and "differential." In the "absolute" method, the detector has a known response to the beta-rays and the activity can be read directly from the effect given by the detector. In the "differential" method, the response of the detector is unknown, but comparison is made using a standard with a known activity and with a radiation identical or closely similar to the P^{32} radiation, both standard and unknown being measured under identical conditions.

The most popular standard for use with the differential method is a radioactive daughter of uranium, namely, UX_2 . In the uranium series, the parent uranium disintegrates by alpha emission with a half-life of 4.6×10^9 years to UX_1 . The UX_1 in turn emits a low energy beta-ray (completely absorbed in ~ 25 mg./cm. $\sim Al$) with a half-life of 24.5 days decaying to UX_2 . (A negligible fraction of UX_1 disintegrates to a nuclear isomer of UX_2 which is known as Uranium Z.) The beta radiation from UX_2 (half-life of 1.14 minutes) has a maximum energy of about 2.3 millions of electron volts and resembles quite closely that from P^{32} . Any sample of reagent uranium or uranium salt is, by virtue of these disintegration relations, in equilibrium with the radioactive daughters of uranium. Thus, a given weight of uranium can be computed to be the equivalent of a certain amount in curies of UX_2 . By spreading a weighed amount of uranium or uranium salt in a dish similar to that used in determining the phosphorus and covering the radioactive substance with sufficient aluminum foil to cut out all but the UX_2 radiation, it is possible to devise a standard with a known activity in microcuries for direct comparison with the P^{32} sample. If a Lauritsen electroscope is used, the amount of uranium needed is large enough so that some UX_2 radiation is lost by self-absorption in the sample and a correction must be applied. Thus, to obtain a microcurie (10^{-6} curies), the quantity of uranium oxide (U_3O_8) required is 3.5 grams. When spread on a dish with an area of 10 cm.² this gives an absorption thickness of 350 mg./cm.² A sample one-tenth as strong as this is a convenient one for use with the electroscope. In this case one must correct for an absorption of 35 mg./cm.² The absorption of the beta-rays is logarithmic, half-value corresponding to approximately 110-120 mg./cm.² in aluminum. The absorption correction must be determined separately in each laboratory since the curve is a function of the particular electroscope and geometry employed. Thus, the scattering of the electrons in the material of the dish and adjacent objects affects markedly the absorption curve obtained. In working with Geiger counters, much thinner samples can be used because of the greater sensitivity of the counters. In any case, a correction for the aluminum foil used to exclude UX_1 radiation is essential.

Such a standard has been employed mainly by the laboratories at the University of California and at Washington University. A recent cross-check of the standards used in these two laboratories shows that agreement within 25 per cent in the absolute value of the microcurie obtains, the St. Louis value

being the lower. Recently, I have found that about two-thirds of this discrepancy can be explained by inhomogeneities in spreading of the P sample with the particular geometry used at St. Louis. Thus, the St. Louis value now agrees with the California microcurie within 10 per cent.

Radiophosphorus supplied by the Radiation Laboratory at Massachusetts Institute of Technology is assayed by the "absolute" method. To obtain a Geiger counter which has a known response to beta-rays, a radioactive substance with known disintegration scheme emitting both beta and gamma rays is measured, using the counter to determine coincidences between beta and gamma rays. By using several such substances and a variety of absorbers, it is possible to determine the variation of counter efficiency with beta-ray energy. This counter, once calibrated in this fashion, is used to determine the counting rate of a Radium E source in equilibrium with its parent 22 year Radium D. This serves as a reference source to check variations in counter efficiency from day to day. The microcurie standard determined in this manner at M.I.T. is smaller than those at either Berkeley or St. Louis by a factor of 2.4. Thus, a sample of radiophosphorus measured in St. Louis as 100 microcuries is measured at M.I.T. as 240 microcuries at present. This discrepancy should be borne in mind when response to therapeutic dosages in different laboratories is compared. The reason for this discrepancy is obscure. Nothing in the present procedures used indicates an error in either method of more than 10 per cent. In preparing samples at Berkeley and St. Louis, an aliquot of the P^{32} solution is pipetted into a bottle cap or porcelain dish, evaporated, and mounted under the detector window. The standard prepared in precisely the same kind of holder and mounted in the same place is measured in identical fashion. The ratio of the two readings gives directly the strength of the P^{32} sample. In the M.I.T. method, the aliquots are pipetted onto paper planchettes, dried, and placed in contact with the counter window. The RaD standards are prepared and assayed in precisely the same fashion. The ratio of the readings corrected for the efficiency of the counter to P^{32} radiation energy gives the strength of the P^{32} sample.*

Investigations are now proceeding at all three institutions to eliminate the discrepancy. Since the prospect of a rapid expansion in radiophosphorus therapy is not remote, it would appear advisable to make available a standardized procedure which is simple and reliable and in which there is agreement among all workers in the field. Prescriptions for precision beta-ray standards resulting from these investigations should make precision standards available.

*The writer is indebted to Prof. J. I. Irvine, Jr., for a description of the method employed at M. I. T.

THE TREATMENT OF ULCERATIVE COLITIS WITH NISULFADINE AND NISULFAZOLE

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ULCERATIVE colitis is a disease of uncertain etiology. In 1931 Hurst stated that in his opinion "the disease is a form of bacillary dysentery." However, some ten years later, after a study of both chronic bacillary dysentery and chronic ulcerative colitis in North China, Snapper stated flatly that "as far as x-ray evidence goes, chronic ulcerative colitis must certainly be distinguished from chronic bacillary dysentery."

In 1924 Bargen reported the presence of a gram-positive diplostreptococcus in 80 per cent of the cases of chronic ulcerative colitis at the Mayo Clinic. He thought this finding of etiologic importance. However, to quote Willard, "Most observers have been unable to confirm Bargen's findings in any large percentage of cases." Paulson, Dukes, Mackie, and Kessel have criticized Bargen's work. Kessel, in his studies on the bacteria found in the colon of normal individuals and in patients with ulcerative colitis, states, "There would, therefore, appear to be no room to regard the streptococci described as Bargen's diplostreptococcus as being essentially different from other types of alpha streptococci commonly recovered from the human alimentary tract and his strains may belong to the species *Streptococcus fecalis*, *Streptococcus mitis*, or *Streptococcus salivarius*."

Another claimant for the role of etiologic agent is the *Bacterium necrophorum*, studied particularly by Daek and his co-workers. Their work is of great interest but awaits further confirmation.

The possible relationship of the virus of lymphogranuloma venereum, or of a similar virus, to ulcerative colitis has been suggested by several observers. Rodaniche, Kirsner, and Palmer in 1940 studied this problem in thirty-four cases of chronic ulcerative colitis. They concluded that "the fact that thirty-two out of thirty-four patients (94 per cent) with chronic ulcerative colitis and proctitis in this series had negative Frei tests and showed no evidence of neutralizing antibodies in their serums against the virus of lymphogranuloma venereum indicates that this virus was in no way involved in the great majority of cases."

The role of psychogenic factors in the etiology of chronic ulcerative colitis has been stressed by many writers. The emotional instability of many of these patients is a fact beyond dispute. However, to assign to it a role beyond that of a predisposing cause would require more proof than has yet been brought forward. Allergy and dietary deficiencies have been suggested by some as

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etiologic factors. Here again, a definite relationship is difficult to prove, although dietary deficiencies are frequently seen in these patients just as they are in other patients suffering from entirely different diseases.

If we find the literature on the subject of the etiology of chronic ulcerative colitis confusing and unconvincing, a review of the methods proposed for the treatment of this disease offers little. The therapeutic procedures proposed (vitamins, artificial fever, oxygen, vaccines, serums, and rectal instillations) are a clear indication that none of them are adequate. The only possible exception is therapy with sulfanilamide and related drugs, where encouraging results have been reported by several observers, notably by Bannick, Brown, and Foster.

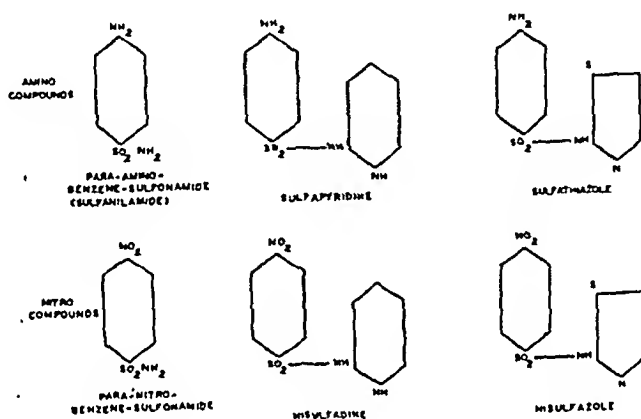


Fig. 1.

During the past four years we have treated twenty-one patients suffering from chronic ulcerative colitis with two new compounds which we have employed in several other studies. These compounds are 2-(p-nitrobenzene sulfonamido)-pyridine, which we shall call nisulfadine, and 2-(p-nitrobenzene sulfonamido)-thiazole, which we shall call nisulfazole. The relationship between sulfapyridine and nisulfadine and between sulfathiazole and nisulfazole is shown in Fig. 1. The physical properties and some of the reactions of these substances have been described in previous papers. A few of these properties may bear a certain relationship to their apparent therapeutic action.*

Nisulfadine is reduced in the intestinal tract to sulfapyridine; nisulfazole, to sulfathiazole. Most of this conversion takes place in the colon and may be the result of bacterial action. In test tube experiments, *Bacillus coli*, *Bacillus typhosus* and *Bacillus dysenteria* (Shiga, Flexner, and Hiss strains) produce a reduction of the nitro compounds when the concentration of these compounds does not exceed 4 mg. per cent. Approximately 50 per cent of the nisulfadine is converted into sulfapyridine and 50 per cent of the nisulfazole is changed to sulfathiazole. When the percentage of the nisulfazole in the broth was increased, it was found that the growth of the Hiss, Shiga, and Flexner strains of *B. dysenteria* was markedly inhibited, while *B. coli* and *B. typhosus* were

*Supplied through the courtesy of George A. Breon & Company, Inc., Kansas City, Mo.

little affected. Both nitro compounds, especially nisulfazole, produced a marked inhibition in the growth of *Streptococcus viridans*, *Str. fecalis*, and *Streptococcus hemolyticus*.

The results of the oral ingestion in dogs are shown in Tables I to IV. When nisulfadine is administered by mouth, approximately 25 per cent appears in the blood as sulfapyridine after four hours (Table I); with nisulfazole, only a faint trace of sulfathiazole appears in the blood after seven hours (Table II). This suggests that nisulfazole is less rapidly reduced and also less readily excreted by the urine. Observations on patients show similar results (Table III).

Our observations on the treatment of chronic ulcerative colitis with nisulfadine and nisulfazole were made on twenty-one patients, the small number of patients treated being due partly to the difficulty of obtaining sufficient supplies of these drugs.

Clinically, all of the patients had shown chronicity and a marked tendency to severe recurring attacks. In each case the clinical diagnosis was confirmed

TABLE I. BLOOD VALUES FOR SULFAPYRIDINE AND NISULFADINE IN THE DOG AFTER THE ORAL ADMINISTRATION OF NISULFADINE 4.35 GM.

BLOOD (HR.)	SULFAPYRIDINE (MG. %)	NISULFADINE (MG. %)
2	Trace	1.13
4	1.02	0.48
8	1.92	0.55
12	3.24	0.30
24	2.79	0.41

TABLE II. BLOOD VALUES FOR SULFATHIAZOLE AND NISULFAZOLE IN THE DOG AFTER THE ORAL ADMINISTRATION OF NISULFAZOLE 5 GM.

BLOOD (HR.)	SULFATHIAZOLE (MG. %)	NISULFAZOLE (MG. %)
2	None	0.59
4	Faint trace	0.75
7	Faint trace	4.30
24	2.0	2.30

TABLE III. BLOOD VALUES OF SULFAPYRIDINE, NISULFAZOLE, SULFATHIAZOLE, AND NISULFADINE IN PATIENTS

DATE	SULFA- PYRIDINE* (MG. %)	NISUL- FADINE (MG. %)	DATE	SULFA- THIAZOLE* (MG. %)	NISUL- FAZOLE (MG. %)
9/ 4/41	5.75	2.90	2/27/42	1.1	6.1
9/ 6/41	4.30	1.50	3/ 3/42	0.7	6.1
9/ 8/41	2.30	2.00	3/ 6/42	0.8	6.7
9/10/41	4.05	1.80	3/11/42	0.8	6.2
9/15/41	4.70	3.60	3/13/42	1.0	5.8
9/17/41	6.35	2.45	3/18/42	1.6	8.4
9/20/41	7.90	3.20	3/21/42	1.5	9.2
Patient received during this period an average dose of 3 Gm. nisulfadine daily			Patient received during this period 4 Gm. nisulfazole daily		

*Values calculated as sulfapyridine and sulfathiazole. Actually known only as compounds containing amino groups giving the characteristic color reaction with sodium nitrite.

by roentgenologic evidence and by the demonstration of ulcers on proctoscopic examination. We were impressed by two complications. First, severe anemia of the secondary type was observed in every patient and could not be explained by the loss of blood from the bowel. Severe anemia, as a concomitant finding in ulcerative colitis, has been stressed by several observers, notably by Schemeusky. Second, four of our patients showed ulcerations on the skin, particularly on the arms and legs. One patient had such deep ulcers on the legs that contractures of the leg muscles appeared, necessitating orthopedic measures. This complication has been studied by many observers. Felsen reported three cases from which he isolated, respectively, *B. coli*, *Staphylococcus aureus* and a nonhemolytic streptococcus. All of our cases began with a small subcutaneous abscess, which gradually increased in size, then burst, discharging thick, creamy pus and leaving an ulcer. The pus, on direct smear, showed no organisms and the cultures made both aerobically and anaerobically, and in a variety of media, showed no growth.

Four of the patients are dead. One patient died of coronary occlusion two years after discharge from the hospital, having had no symptoms of colitis during the interval. Another patient died one month after cessation of all intestinal symptoms, the cause of her death being unknown. A third patient died from lobar pneumonia two years after dismissal from the hospital, apparently well. A fourth patient who also suffered from bronchiectasis died six months after dismissal from the hospital, the cause of death being probably pneumonia complicated by recurrence of ulcerative colitis. One patient was operated upon and an ileostomy performed. Under treatment with nisulfazole the number of stools before operation was reduced from a daily average of from eighteen to two.

All of the patients treated were greatly improved, including those who subsequently died. Seventeen of these patients are now free from symptoms and show normal roentgenograms; seven are markedly improved, four of these being still under observation and receiving treatment from time to time. The drug first used was nisulfadine, but later nisulfazole was employed exclusively since it produced less nausea. The initial dose was from 4 to 6 Gm. in twenty-four hours; with clinical improvement, this dose was reduced to 2 Gm. Reduction in the number of bowel movements was prompt and striking. In five patients, after two weeks of therapy, enemas were occasionally necessary to produce bowel movements. Prolonged medication over a period of months caused no untoward symptoms, although in several instances a reduction in the leucocyte count led us to discontinue medication for a brief interval.

The drug was administered in three forms. The patients received first the compound in tablets. In some instances the tablets soon produced nausea and in their place a pectin suspension of nisulfadine was employed. This was usually tolerated by the patient. In some patients, nisulfazole in a pectin suspension was administered by rectum. This method of administration was usually promptly effective and produced no nausea. More recently, enteric-coated tablets of nisulfazole have been employed. These are effective and apparently produce no nausea. However, while the patient has diarrhea, they

may pass through the intestinal tract unchanged because of the active peristalsis and are usually ineffective during the first days of the treatment. The method of administration may vary with the individual and a decision as to the best method awaits further experience.

In addition to drug therapy, the patients were placed on a diet with a minimum of residue and received vitamin therapy, especially B complex, and those with marked anemia were given blood transfusions.

In Table IV are given data on the patients studied in the Medical Clinic of the University of Kansas.

A few additional remarks should be made regarding the data given in Table IV. The term recovery is employed when the patient is symptom free and the x-ray picture shows a normal colon. The term improvement is employed to designate patients who have no diarrhea and have normal stools but show stenosis on thickening of the colon in the x-ray picture. Patients 14, 18, 19, and 20 are symptom free at present but still show a narrowed and constricted colon. These patients had suffered from chronic ulcerative colitis thirteen years, ten years, eighteen years, and twelve years, respectively. These changes in the colon are probably permanent.

The term slight relapse is employed in four instances where the patient developed a slight diarrhea and following instructions returned promptly to the hospital for treatment, to which they responded promptly.

The following are two typical case histories:

D. B., aged 37 years, was admitted to the University of Kansas Hospitals Aug. 4, 1942, complaining of diarrhea. The family history was negative. Past history: the patient had enjoyed good health up to the onset of the present illness, which began approximately six years before admission to the hospital, and since that time had had continued diarrhea which had resisted all types of therapy. The diarrhea had increased in severity through the years. During the past two months the diarrhea had been particularly severe, the patient having never less than eight and often as many as twenty stools in twenty-four hours.

Physical examination showed evidence of marked loss of weight: Proctoscopic examination showed mucous bleeding and numerous ulcerations in the rectum. The patient weighed 111 pounds. Admission blood count was 3,600,000 red cells, 6,700 white cells, and 68 per cent hemoglobin (10.5 Gm.). X-ray of the colon showed a colon devoid of haustrations, varying greatly in diameter, being narrow in the region of the splenic flexure and wide in the transverse and descending colon. There was a granular type of architecture throughout the colon from the hepatic flexure downward and feathering of the margin of the colon, and the conclusion of the roentgenologist was ulcerative colitis involving the transverse and descending colon and sigmoid.

The patient was started on nisulfazole, 1 Gm., six times daily, which was later reduced to four times daily. In addition to this treatment, she was given six blood transfusions during her stay in the hospital, which lasted to Oct. 11, 1942. At the time of her dismissal, she was very markedly improved, the stools within ten days having been reduced to not more than two in twenty-four hours.

The patient was followed further and has continued to improve since her dismissal from the hospital. On Dec. 13, 1942, she was readmitted for an acute abdominal condition, diagnosed as acute hemorrhage into round ligament and abdominal wall.

On May 11, 1945, she was readmitted to the hospital for observation. The blood count at this time was 4,120,000 red cells, 4,200 white cells, and 74 per cent hemoglobin. X-ray of the colon showed no narrowing of the colon and no evidence of ulceration. There

TABLE IV

PATIENT	AGE (YR.)	SEX	DURATION	ADMITTED	NUMBER OF STOOLS DAILY ON AD-MIS- SION		LENGTH OF TREATMENT	RESULT	RELAPSE	REMARKS
1	71	M	18 mo.	6/23/41	10	2	1 mo.	Recovery	None	Died, "heart attack," 10/19/43
2	16	M	4 mo.	6/30/41	18	2	3 wk.	Recovery	One slight	Died, pneumonia, 1/18/44
3	23	M	14 mo.	12/14/41	14	2	5 mo.	Recovery	Two slight	Well, 2/15/44
4	19	F	9 mo.	1/19/42	10	2	4 wk.	Recovery	One slight	Well, 11/23/45; gained twenty pounds
5	22	F	5 mo.	2/13/42	27	3	2 mo.	Recovery	None	Died later, cause unknown; no diarrhea
6	19	M	30 mo.	7/23/42	8	3	3 mo.	Improvement	Two	Died, 12/21/44
7	37	F	12 mo.	8/ 4/42	20	2	10 wk.	Recovery	None	Well, 5/11/45; three pounds
8	18	F	6 yr.	1/16/43	7	1	1 mo.	Recovery	None	Well, 1/14/44
9	54	M	3 yr.	1/19/43	18	2	4 mo.	Improvement	None	Operation
10	40	M	5 mo.	8/ 2/43	8	2	1 mo.	Recovery	None	"Perfectly well," 8/24/45
11	41	F	1 mo.	10/24/43	10	1	10 wk.	Recovery	None	Well, "feeling fine," 5/18/45
12	37	F	4 mo.	3/14/44	6	1	3 mo.	Recovery	None	Well, 11/5/45; gained thirty pounds
13	24	M	18 mo.	7/20/44	10	2	2 mo.	Improvement	None	Improved, 9/24/45
14	13	M	5 yr.	8/22/44	12	2	1 mo.	Improvement	One	Improved, 10/21/45
15	30	F	3 yr.	10/22/44	15	2	2 mo.	Recovery	None	Well, 10/2/45
16	18	F	7 mo.	10/31/44	18	1	2 mo.	Recovery	One slight	Well, 1/18/46
17	27	F	4 yr.	11/25/44	20	2	4 mo.	Recovery	None	Well, 8/1/45; gained thirty pounds
18	14	M	10 yr.	1/24/45	8	2	1 mo.	Improvement	Two	Continued improvement, 10/1/45
19	55	M	18 yr.	3/ 1/45	8	2	7 wk.	Improvement	None	Well, 10/15/45; gained ten pounds
20	24	F	12 yr.	3/10/45	7	2	5 wk.	Improvement	None	Well, 10/1/45; gained fifteen pounds
21	44	M	11 yr.	4/14/45	4	1	3 wk.	Recovery	None	Well, 9/18/45

was still some lack of haustrations although this was not marked. The patient has continued to be free of diarrhea and weighs 164 pounds, a gain of fifty-three pounds since the time of her first admission to the hospital.

T. M., a Negro woman, was admitted to the University of Kansas Hospitals March 14, 1944, complaining of bloody diarrhea. The family history was negative. Past history: the patient had enjoyed general good health and had had no severe illnesses; described herself as being of a somewhat nervous disposition. The present illness began in December, 1943, when the patient began having bloody diarrhea with from eight to ten movements a day. This continued until admission to the hospital. She had become very anemic and lost fifteen or twenty pounds (weight now, 110 pounds).

On admission to the hospital the patient showed evidence of loss of weight; the mucous membranes were rather pale. The heart, lungs, and abdomen were negative on admission. The blood pressure was 85/65 and the pulse, 120. Examination of the rectum ten days after admission to the hospital showed numerous small ulcers.

On admission, the blood count showed 2,850,000 red cells, 13,000 white cells, and 55 per cent hemoglobin. Urinalysis was negative. X-ray examination showed complete lack of haustrations in the colon; along the medial contour of the descending colon definite feathering was seen, indicative of superficial ulceration. The conclusion of the roentgenologist was "active ulcerative colitis."

Treatment was started immediately and she received nisulfazole, 4 Gm. daily, with vitamin C, 1 Gm. In addition to this, she was given blood transfusions, five being given during her entire stay in the hospital.

Improvement was noticeable within forty-eight hours. The patient's bowel movements were reduced to three a day, and from March 20 until the time of her dismissal, the bowel movements never exceeded three in twenty-four hours.

At the time of her dismissal from the hospital May 24, 1944, the blood count was 4,190,000 red cells, 7,650 white cells, and 74 per cent hemoglobin. The report on the barium enema on that day was: "The colon fills normally. No pathology is seen. No evidence of ulceration is seen; neither is there scarring."

This patient has been followed since and continues in excellent condition. After leaving the hospital she continued taking 2 Gm. of nisulfazole daily, which was discontinued July 14, 1944. The blood count June 2, 1945, was 4,240,000 red cells, 6,000 white cells, and 81 per cent hemoglobin.

The patient's condition at the present time is excellent. A letter of Nov. 3, 1945, states: "I have one bowel movement a day; once in a while I won't have any."

In a disease such as chronic ulcerative colitis, a disease noteworthy for its chronicity and its tendency to recurrence, it would be hazardous to assert that most of these patients are permanently cured. We do feel, however, that therapy with nisulfazole has given us much better results than any other treatment with which we are familiar.

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OBSERVATIONS ON THE TREATMENT OF TROPICAL SPRUE WITH FOLIC ACID

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THE discovery of the antianemic effect of synthetic folic acid in certain types of macrocytic anemia in relapse opens up a new era in the study of the pathogenesis and therapy of these conditions.¹⁻³ Included in the group of patients so dramatically relieved by folic acid were those with Addisonian pernicious anemia and those with nutritional macrocytic anemia. Because the pathogenesis of none of the macrocytic anemias is thoroughly understood, there has been much confusion concerning their diagnosis. Our studies^{4,5} have shown that Addisonian pernicious anemia and nutritional macrocytic anemia are cytologically indistinguishable and that the best single differentiating feature is the presence of free hydrochloric acid in the gastric juice in persons with nutritional macrocytic anemia and the absence of free hydrochloric acid in the gastric juice of persons with Addisonian pernicious anemia even after histamine stimulation. We have found that the macrocytic anemia of pellagra and of pregnancy, from a laboratory point of view, are in no way distinguishable from nutritional macrocytic anemia, and all respond to folic acid. It seems wise, therefore, to abandon the terms "macrocytic anemia of pellagra" and "macrocytic anemia of pregnancy" and classify them as nutritional macrocytic anemias. We have also observed, as have many others, that cytologically the anemia of sprue and nutritional macrocytic anemia are identical. The relationship between the two diseases is very difficult to grasp, and differentiation may not be justifiable. The final answer to the question may have to wait until their etiology is more completely understood. Acid steatorrhea is a characteristic and specific feature of sprue, and we make a diagnosis of sprue rather than nutritional macrocytic anemia in the presence of steatorrhea.

Although macrocytic anemias occur throughout the world, certain types are found much more frequently in one area than in another. In the temperate zones, only sporadic cases of so-called nontropical sprue are seen, whereas in some of the tropical areas sprue is endemic. There is a difference of opinion among some physicians as to the pathogenesis of tropical and nontropical sprue, but we consider both as similar conditions. The number of cases of sprue seen in

From the University of Cincinnati Studies in Nutrition at the Calixto Garcia Hospital, Havana, Cuba, in cooperation with the Institute of Nutrition of Cuba and the University of Havana.

In the medical care of the patients in the special ward, we were assisted by many physicians who aided us throughout the course of the study. We are especially indebted to Dr. Aureliano Rodríguez and Dr. Carlos Castellanos, for the gastric analyses, and to Dr. Juan Bencomo and his assistants, for the blood chemistry determinations. The determination of the fat was done by Dr. Emilio Morales, and the bacteriology by Professor Arturo Curbelo. Parasites were studied and identified by Prof. P. Kouri, and retroscopic examinations were done by Dr. Aureliano Rodríguez and Dr. Carlos Castellanos.

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the United States, however, is small; and, in order to study thoroughly the effectiveness of folic acid in treating the macrocytic anemia of tropical sprue, it seemed necessary to go to an area in the tropics. Through the cooperation and assistance of the University of Havana and the Institute of Nutrition of Cuba, a special ward was obtained at the University Hospital in Havana. Twenty-five patients were examined clinically and hematologically, and of this number nine were selected for the study.

SELECTION OF PATIENTS

In selecting the patients for the therapeutic assay of folic acid, the following criteria were used: (1) The patient had to have a macrocytic anemia; (2) the bone marrow had to show the typical erythroblastic arrest seen in pernicious anemia; (3) the erythrocyte counts had to be below 2.5 million; (4) the patient had to be untreated, or he must not have been treated recently enough to interfere in any way with our evaluation of the effect of folic acid; (5) he had to have persistently low reticulocyte counts during the period of observation; (6) he had to have glossitis and diarrhea characterized by fatty stools.

With these criteria in mind, we selected for study nine white patients (six men and three women) ranging in age from 29 to 75 years.

METHOD OF STUDY

All the patients were hospitalized for preliminary examination, base-line determinations, and treatment. A detailed medical and dietary history was taken and a complete physical examination was made in each case. From the time the patients were accepted for study, their diets were rigidly controlled. Meat, meat products, fish, and poultry were excluded, and only one quart of milk and one egg were allowed daily. Bread, cereals, fruits or vegetables, sugar, fats, and coffee were allowed in any amount desired. In a previous study of anemia, seventy-five patients were restricted to this type of diet, and none of them had a so-called spontaneous remission. We felt reasonably certain, therefore, that any hemopoietic response would be attributable to the folic acid rather than to any food the patients received.

Daily hematologic examinations included white and erythrocyte counts, hemoglobin determinations, and reticulocyte counts. Certified Trenner pipettes were used for both the white and erythrocyte counts. The hemoglobin content of the blood was determined in grams by means of a Leitz colorimeter, and the reticulocytes were counted in wet preparations by the use of a modified brilliant cresyl blue solution of Dameshek. From time to time cell volumes were determined on oxalated venous blood by means of Wintrobe hematocrit tubes. In each case bone marrow was obtained prior to treatment by sternal aspiration. Differential counts were made on preparations stained with both supravital and Wright-Giemsa stains. Gastric contents were collected before and after histamine injection in all patients.

In each case a twenty-four hour stool specimen was collected daily in a single large glass graduated container. The stools were examined every day for appearance and volume. Each patient was questioned every morning con-

cerning the number of bowel movements. Examinations of the feces included tests for acidity, fatty acid determinations by the method of Labbé and Larué, bacteriologic examinations, and examinations for parasites.

Serum protein determinations were made in each patient. In most cases blood calcium, phosphorus, potassium, amylase, and lipase determinations were made.

Dietary histories revealed that in each case the diet had been deficient in animal protein over a long period of time. The foodstuffs containing animal protein most frequently ingested were milk and occasionally eggs, but these were always consumed in inadequate quantities. Vegetables were rarely eaten and the only fruits eaten were mangos, avocados, and citrus fruit, and these only in small amounts. The diet consisted almost entirely of rice, corn meal, and *viandes* (root vegetables grown in Cuba).

Loss of body weight, which had occurred in all subjects, varied from fifteen to forty pounds. All patients complained of extreme weakness and fatigue on even slight exertion. During the preliminary period of observation, they lay quietly in bed. They had no apparent desire to move and manifested no interest in their surroundings. All the patients except one (Case 8) complained of loss of appetite and a distaste for food. Without exception, they complained of a feeling of fullness, flatulence, and abdominal distention after meals and of a burning sensation of the mouth and tongue which increased in severity while they were eating. This sensation sometimes extended to the throat and epigastrium after swallowing. In some patients rectal burning was described.

All the subjects gave a history of having had diarrhea for periods of time which ranged from four months to four years. The stools were light colored, large (from 200 to 1,200 c.c. a day), frothy, foul smelling, semiliquid or liquid, and varied in number from three to twenty a day. Bowel movements were accompanied by expulsion of large amounts of gas and by borborygmus. In many instances the bowel movements were preceded by colic.

Physical examinations revealed that in each case the skin was pale and dry with desquamating areas which varied in location in different patients but appeared most commonly on the arms, hands, and face. In some patients pigmentation was generalized. In four patients there were hemorrhagic diffusions of violet discoloration on the dorsum of the hands and forearms. Glossitis, either diffuse or localized, was present in every patient and was accompanied by atrophy of the papillae. In some patients imprints of the teeth could be seen along the margins of the tongue and in some individuals the tongue showed hemorrhagic suffusions on the inferior aspect. Cheilosis was present in two patients (Cases 1 and 8). In every subject the hair was dry and brittle.

Numbness and tingling of the hands and feet were complaints common to all patients. Four of the patients complained of dizziness when standing. Other abnormal neurologic signs were found in only two of the subjects. One (Case 2) had pain on pressure of the muscles of the legs. She also complained of sciatic pain. These symptoms were relieved with thiamine. Another (Case 3) presented a flaccid paraplegia, and the tendon reflexes were absent. She had a history of syphilis with positive serology as determined by Kahn and Meinicke

tests. The spinal fluid examination showed a syphilitic gold curve and an increase in globulin content.

Seven patients had dependent pitting edema. The five patients above 50 years of age had arteriosclerosis of varying degree of severity. Abdominal examinations showed meteorism, slight distention, and softness to palpation in all cases. In one patient (Case 3) there was ascites. The liver and spleen were not enlarged in any of the patients. Aside from emphysema in two subjects, there were no abnormal thoracic findings. Rectoscopic examinations were performed in six subjects. The mucosa of the rectum showed variable degrees of atrophy, paleness, and slickness.

TABLE I. BLOOD INDICES

CASE	PATIENT	MEAN CORP. VOL. (CU. MICRONS)	MEAN CORP. HB. (MICRO MICROGRAMS)	MEAN CORP. HB. CONC. (%)
1	C. G.	128	45	38
2	M. S.	105	36	34
3	C. B.	145	46	32
4	J. C. L.	134	44	30
5	G. A.	117	44	38
6	A. M.	131	47	36
7	A. F.	130	44	34
8	P. E.	119	42	32
9	J. G.	142	49	35

All the patients had macrocytic anemia with mean corpuscular volumes of from 105 to 145 cubic microns, mean corpuscular hemoglobins of from 34 to 47 micro micrograms, and mean corpuscular hemoglobin concentrations of from 30 to 38 per cent (Table I). The erythrocyte counts ranged from 1.47 to 2.28 million per cubic millimeter. The red blood cells showed marked anisocytosis, poikilocytosis, and polychromasia. Five subjects (Cases 2, 4, 5, 7, and 9) had leucocyte counts below 5,000 per cubic millimeter but only one (Case 5) had a count less than 3,000. The hemoglobin values ranged from 5.6 to 10.1 Gm. per 100 c.c., and the initial reticulocyte counts were less than 2.7 per cent. Bone marrow differentials showed the erythroblastic arrest characteristic of pernicious anemia, but in general there was only a 3 to 2 or a 2 to 1 predominance of myeloid over erythroid elements (Table II). In one patient only (Case 5) was there a 1 to 1 distribution of nucleated red cells to white cells.

Gastric analyses showed none of the cases to be histamine refractory, but, on the contrary, the patients were found to have normal or increased gastric acidity (Table III).

TABLE III. GASTRIC ANALYSES

CASE	PATIENT	FASTING		AFTER HISTAMINE	
		FREE°	TOTAL°	FREE°	TOTAL°
1	C. G.	60	70	65	80
2	M. S.	60	70	50	60
3	C. B.	0	10	40	50
4	J. C. L.	0	10	30	40
5	G. A.	0	12	90	100
6	A. M.	40	50	95	100
7	A. F.	0	15	45	50
8	P. E.	15	20	65	75
9	J. G.	30	45	115	130

TABLE II. DIFFERENTIAL BONE MARROW COUNTS

CASE	PATIENT	BASOPHILES (PER CENT)	EOSINOPHILES (PER CENT)	NEUTROPHILES (PER CENT)	LYMPHOCYTES (PER CENT)	SEGMENTED (PER CENT)	LYMPHOCYTES (PER CENT)	CLASMATOCYTES (PER CENT)	MEGAKARYOCYTES (PER CENT)	PLASMA CELLS (PER CENT)	RETICULUM CELLS (PER CENT)	NORMOBLASTS (PER 100 W.B.C.)	LATE ERYTHROBLASTS (PER 100 W.B.C.)	EARLY ERYTHROBLASTS (PER 100 W.B.C.)	MEGALOBLASTS (PER 100 W.B.C.)	NUMBER OF NUCLEATED R.B.C./100 W.B.C.
1	C.G.	0	3	C-27 0.27	20	19	18	8	3	1	0	6	19	15	2	42
2	M.S.	0	3	D-2 0.23	18	10	10	33	1	0	0	25	12	8	2	47
3	C.B.	0	2.5	C-20.5 B-1	15.5	12.5	23.5	16	1.5	0	0	15	18	16	5	51
4	J.C.L.	0	2.5	C-17 B-0.5	17	7.5	32.5	20.5	0.5	0	2	28	24.5	20.0	15.5	97
5	G.A.	0	1	C-37 B-2	28	7	9	14	3	0	0	23	10	16	11	60
6	A.M.	0	6	C-13.5 B-1.5	17	5	30	23.5	3.5	0	0	12	35	17	3	67
7	A.F.	0	1	C-27 B-3	28	11	10	20	0	1	0	12	22	7	0	41
8	P.E.	0	3	C-17 B-3	17	17	26	13	1	1	0	4	12	15	4	40
9	J.G.	0	2	C-10	10	10	49	17	1	0	0	34	17	4	4	59
<i>After Folic Acid Administration</i>																
1	C.G.	0	1	C-25	12	14	25	17	5	1	0	71	13	3	0	87
8	P.E.	0	13	C-12 C-18	8	16	31	18	1	1	0	109	7	5	0	121
9	J.G.	0	1	B-1	8	10	26	32	2	1	0	117	4	2	0	123

TABLE IV. BLOOD CHEMISTRY

CASE	PATIENT	SERUM PROTEINS				CHOLESTEROL		AMYLASE (UNITS PER 100 C.C.)	LIPASE (C.C. 1/20N NROH PER 100 C.C.)	CALCIUM (MG./100 C.C.)	PHOSPHORUS (MG./100 C.C.)	POTASSIUM (MG./100 C.C.)
		TOTAL (GM./100 C.C.)	ALBUMIN (GM./100 C.C.)	GLOBULIN (GM./100 C.C.)	FIBRINOGEN (MG./100 C.C.)	TOTAL (MG./100 C.C.)	ESTERS (MG./100 C.C.)					
1	C. G.	4.2	2.4	1.8								
2	M. S.	4.0	2.5	1.5	600	306	169					
3	C. B.	4.6	2.5	2.1	400	190	99	108	1.0	11.5	3.0	16.0
4	J. C. L.	5.0	2.8	2.2	400					11.0	4.2	13.0
5	G. A.	3.3	1.9	1.4		285	148	109	2.0	11.5	3.5	13.5
6	A. M.	5.0	2.2	2.4	400	203	128	110	0.3	12.0	3.5	10.0
7	A. F.	3.6	1.9	1.7		230	120	100	2.0	12.0	4.0	16.0
8	P. E.	5.4	3.6	1.8		225	180	98	1.3	10.8	5.0	13.4
9	J. G.	5.5	3.1	2.4	400	187	110	102	0.7	12.0	4.0	16.0

In all patients tested, the blood calcium, phosphorus, potassium, amylase, and lipase values were normal (Table IV). The total cholesterol was normal or moderately increased, with the cholesterol ester ratio to total cholesterol moderately decreased. Serum proteins were as a rule low, from 3.3 to 5.4 Gm. In only two cases was the ratio of albumin to globulin over 1.5. In seven patients it was from 0.9 to 1.3. Serum iron determinations were performed in six instances. The values ranged from 83 to 247 micrograms of iron with an average of 156 micrograms. Only two values (197 and 247 micrograms) were above the normal range (from 50 to 180 micrograms).⁶

No gastroscopic examinations were performed in this series; however, in a similar series of twenty-five cases found in Cuba, Milanés reported that most of the patients had localized areas of atrophic and hypertrophic gastritis and a few had diffuse atrophic gastritis.⁷

A study of the feces showed that the reaction was acid in seven cases. In these the values for fatty acid determined by the method of Labbé and Larué were above 11 c.c. of 0.1 N sodium hydroxide. The amounts were well above the normal range (from 0 to 7 c.c. of 0.1 N sodium hydroxide). (Table V.) The special determinations of total fats, neutral fats, and other information will be reported separately. The starch residue was above normal. Bile pigments were present in normal amounts. The feces in four subjects (Cases 1, 3, 5 and 6) were infested with *Trichuris trichiura*. The feces of cases 3 and 7 contained ova of *Ascaris lumbricoides*. One subject (Case 1) also expelled one *Ascaris lumbricoides*, but several serial examinations were negative for ova. Another patient (Case 6) had *Giardia intestinalis* diagnosed in material obtained by duodenal intubation. The feces of this patient also contained ova of *Necator americanus*. In each case bacteriologic examination of the stools and of material obtained by curettage of rectal mucosa showed that pathogens were not present. Samples obtained from the small intestine through a Miller-Abbott tube yielded no pathogenic bacteria.

TABLE V. STOOL ANALYSES

CASE	PATIENT	pH	FATTY ACIDS C.C./0.1 N NaOH (PER 100 GM. MOIST FECES)	PATHOGENS AND PARASITES
1	C. G.	6.0	16.0	Ova of <i>Trichuris trichiura</i> , <i>Ascaris lumbricoides</i>
2	M. S.	4.5	12.0	None
3	C. B.	7.5	11.0	Ova of <i>Trichuris trichiura</i> ; ova of <i>Ascaris lumbricoides</i>
4	J. C. L.	5.5	12.3	None
5	G. A.		14.2	Ova of <i>Trichuris trichiura</i>
6	A. M.	7.5	11.2	Ova of <i>Trichuris trichiura</i> , <i>Necator americanus</i> , <i>Giardia intestinalis</i>
7	A. F.	6.5	15.0	Ova of <i>Ascaris lumbricoides</i>
8	P. E.	4.5	12.3	None
9	J. G.	5.5	17.8	None

RESULTS FOLLOWING ADMINISTRATION OF FOLIC ACID

When all the preceding findings were obtained, each of the nine patients was given folic acid, orally administered in water suspension between meals. The folic acid was weighed on an analytic balance. Six subjects (Cases 1, 2, 5, 7, 8, and 9) were given 100 mg. twice daily. Subsequent experience having indicated that the daily dosage of 200 mg. was more than necessary, the daily dose was decreased to 100 mg. after the patient has passed the peak of his reticulocyte response. Three subjects (Cases 3, 4, and 6) received 10 mg. of folic acid per day. One subject (Case 2) received, in addition, 100 mg. of thiamine chloride daily, intramuscularly and another (Case 7) received, in addition to the folic acid, 100 Gm. of a yeast extract* each day; on one occasion the latter patient was given 250 c.c. of plasma intravenously to reduce the amount of edema present. Two laboratory workers, who had red counts of 5.15 and 5.24 millions per cubic millimeter and hemoglobins of 15.8 and 16.2 Gm., respectively, received 200 mg. each of folic acid, daily for ten days, and served as controls.

The response of the patients receiving 10 mg. of folic acid has so closely paralleled the response of those receiving 200 mg. that they will not be discussed separately. With the exception of one subject (Case 2) who had a deficiency of both niacin and thiamine, and another (Case 7) who had anasarca, all the patients began to recover strength and to experience a feeling of well-being within three or four days after the initiation of folic acid therapy. The response of these two patients to folic acid was similar but perhaps retarded somewhat. In all cases the pigmentation of the skin decreased. In one patient (Case 9) who has been treated with folic acid for forty-one days the skin has cleared almost entirely. The patients have not been outside the hospital since their admission, so that this may be due in part to lack of sunshine. In every subject there was a striking change in the facial expression, which became alert and lively, whereas prior to treatment, it had been almost masklike. The con-

*Basamin, Anheuser-Busch, St. Louis, Mo.

junctional mucosa changed from intense paleness to varying degrees of recoloration which was in direct proportion to the increase in blood hemoglobin and erythrocyte values.

Three representative cases are reported briefly; they give the reader an idea of the striking improvement effected by folic acid.

CASE 4.—J. C. L., a 63-year-old white man, was admitted to the Calixto Garcia Hospital Nov. 19, 1945, complaining of diarrhea, weakness, and dizziness.

Throughout his adult life his diet had consisted chiefly of root vegetables and corn meal. Occasionally he ate an orange or a piece of pineapple and once or twice a month he ate a piece of beef. Although he noticed that he was losing strength some years ago, he dates the onset of his present illness to seven months before admission to the hospital. At this time he noticed burning and soreness of the tongue and small sores in the mouth. Almost simultaneously he began to pass very large bulky yellow or white stools. His appetite became so poor and he became so weak that he consulted his physician in Cienfuegos, who referred him to us for treatment.

THE EFFECT OF FOLIC ACID ON THE MACROCYTIC ANEMIA OF SPRUE

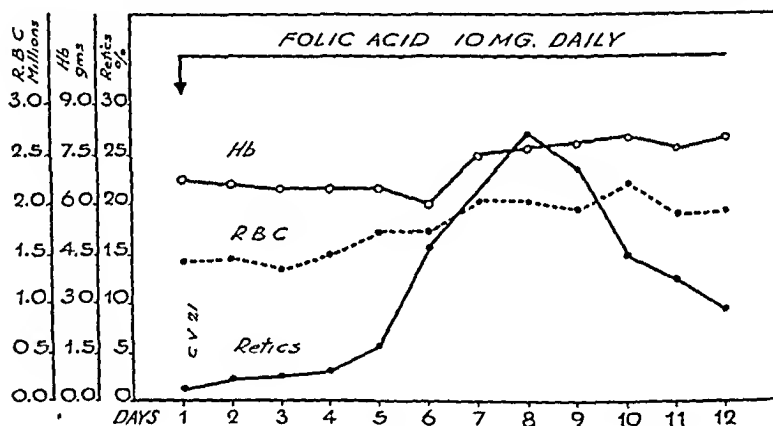


Fig. 1.

Physical examination showed an extremely emaciated, pale, apprehensive man. By the time he came to us for treatment we had learned that the administration of 10 mg. of folic acid daily by mouth was followed by a positive response. Accordingly, he was given this dosage. His hemopoietic response is shown in Fig. 1. Within ten days after treatment was initiated, the profuse liquid, light-colored stools became normal in color; they were solid and much smaller in volume. Since then, the stools have been copious and semisolid on some days, and other days they have been well formed and small in volume. His appetite at the present time is tremendous. He has no alimentary tract symptoms, his strength has returned, and he is eager to return to work.

CASE 5.—G. A., a 62-year-old man, was admitted to the Calixto Garcia Hospital Nov. 22, 1945, complaining of diarrhea, burning of the esophagus, and asthenia.

He considered that his health had been perfect until the onset of the present illness four months before admission to the hospital, despite the fact that during all his adult life his diet had consisted chiefly of rice, potatoes, corn meal, root vegetables, avocados, and occasionally a glass of milk, an egg, or a small serving of pork. The onset of his illness was sudden and was preceded only by "indigestion" and gastric distress which, within a

few days, was followed by loss of appetite and severe diarrhea. He had from eighteen to twenty foul-smelling stools daily which were frothy and sometimes yellow in color and sometimes "like clear water." Although he passed no blood, he sometimes had severe burning of the rectum. A few days after the onset of his illness he became so weak and dizzy that it interfered with his work. During the four months he was ill he lost thirty-eight pounds in weight.

Physical examination showed a small, pale, emaciated man. The buccal mucosa was extremely pale as was the tongue except where it was bright red at the borders and tip. The papillae were smooth and the tongue was edematous at the border so that the imprint of his teeth was clearly visible. Dependent pitting edema over the dorsum of the feet extended halfway up the legs and made it impossible for him to raise his feet or put on his shoes.

THE EFFECT OF FOLIC ACID ON THE MACROCYTIC ANEMIA OF SPRUE

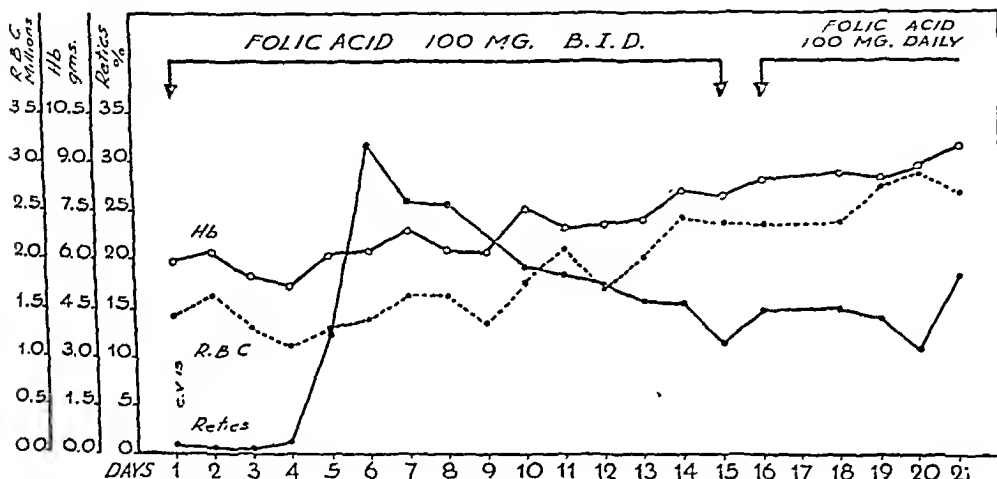


Fig. 2.

He was given 100 mg. of folic acid twice daily by mouth. Two days later his appetite became tremendous and the soreness of the tongue disappeared. Soon after this the stools became semisolid and on some days were well formed and normal in color. Frequently he had only one or two stools daily and often they amounted to only 100 c.c. in volume. The edema has disappeared, and he says that he feels stronger than he has felt for years and that he wishes to return to work. His hemopoietic response to treatment is shown in Fig. 2.

CASE 8.—P. E., a 63-year-old man, was admitted to the Calixto Gareia Hospital Nov. 19, 1945, complaining of diarrhea and weakness.

During most of his adult life he had been unable to eat pork or beef because they caused gastric distress. He rarely drank milk. For a number of years his diet had consisted chiefly of potatoes, root vegetables, corn in various forms, and from three to four eggs daily. Other than the gastric distress following the ingestion of beef and pork, his health had been perfect until five years before admission to the hospital, when he noticed a burning sensation of the mouth and tongue and swelling of the tongue. Soon after this he began having severe diarrhea. Sometimes he had thirty bowel movements daily which were usually preceded by severe colic. The stools were yellow in color and foamy. He consulted a physician who gave him liver extract which he took intermittently for three years, during which time he remained well. A year and one-half after he discontinued taking liver extract, he again began having diarrhea, and all the symptoms he had during his first illness reappeared. He

lost his appetite, began having paresthesias of the legs, dizziness, and tinnitus and became progressively weaker. After losing twenty pounds in weight he reported to his physician, who sent him to us.

Physical examination showed a well-developed, pale, emaciated man who lay quietly in bed with no apparent desire to move. The tongue was slick and shiny and was intensely red along the tip and edges.

He was given 100 mg. of folic acid twice daily by mouth. Three days after this therapy was initiated, his appetite, strength, and vigor greatly increased. Prior to treatment the volume of the stools had been about 1,000 c.c. daily, and they had been light in color and frothy (see Figs. 3 and 4). The volume gradually decreased and the color became more normal. Although the stools have not become completely normal, he says he feels stronger than he has felt for many years and that he wishes to return to work. His hemopoietic response to folic acid is shown in Table VI (Case 8).



Fig. 3.

Fig. 3.—Photograph taken before folic acid therapy. Note consistency and copious amount of the stools.



Fig. 4.

Fig. 4.—Photograph taken ten days after folic acid therapy was begun. The amount of fecal material has decreased greatly, the stool is no longer liquid, and it is more nearly normal in color.

The hematologic data are presented in Table VI. Following therapy with folic acid, reticulocytosis occurred in every instance with peak values of from 12.5 to 31.8 per cent on 200 mg. daily, and from 18.8 to 26.2 per cent on the 10 mg. dosage given to three of the patients (Cases 3, 4, and 6). In all cases the response was maximal according to the Minot and Castle formula based on the initial erythrocyte count.⁸ The day of the peak ranged from the sixth

TABLE VI. ANTIANEMIC EFFECT OF FOLIC ACID IN SPRUE

CASE	PATIENT	RED CELLS (MILLIONS/C.MM.)			HEMOGLOBIN (GM./100 C.C.)			RETICULOCYTOSIS			FOLIC ACID (MG. PER DAY)	NUMBER OF DAYS OF AD- MINIS- TRATION
		INI- TIAL	14 DAYS	FINAL (DAY)	INI- TIAL	14 DAYS	FINAL (DAY)	INI- TIAL %	DAY OF PEAK	% AT PEAK		
1	C. G.	1.47	2.55	3.82 (37)	6.8	8.6	11.9 (37)	1.4	6	22.7	200 100	22 15
2	M. S.	1.99	2.60	2.96 (32)	7.2	10.0	10.8 (32)	2.6	6	19.5	200 100	15 17
3	C. B.	1.52	2.40	2.40 (14)	7.1	10.7	10.7 (14)	1.6	8	22.6	10	14
4	J. C. L.	1.57	2.03	3.20 (23)	7.0	7.4	10.7 (23)	1.1	7	26.2	10	23
5	G. A.	1.28	2.49	3.24 (32)	5.6	8.1	10.7 (32)	0.8	6	31.8	200 100	15 17
6	A. M.	2.13	2.84	3.45 (18)	10.1	11.7	12.4 (18)	0.8	8	18.8	10	18
7	A. F.	2.16	2.66	3.05 (41)	9.5	9.7	11.3 (41)	1.6	7	17.2	200 100	24 17
8	P. E.	2.28	2.85	3.93 (31)	8.3	10.0	11.0 (31)	1.1	8	12.5	200 100	15 16
9	J. G.	1.97	3.04	3.94 (41)	9.7	10.4	13.8 (41)	1.9	7	17.2	200 100	25 16

to the ninth. The red cell counts rose from 0.50 to 1.21 million per cubic millimeter in fourteen days in the patients receiving 200 mg. of folic acid. It increased from 0.46 to 0.71 million per cubic millimeter in the patients receiving 10 mg. doses. (See figures in representative case histories.) One subject (Case 5) was given 200 mg. of folic acid each day for fifteen days and 100 mg. daily for seventeen days. He had a reticulocyte response of 31.8 per cent on the sixth day. Another (Case 4, Fig. 1) was given 10 mg. of folic acid each day. His initial erythrocyte count was 1.57 million per cubic millimeter. His reticulocytes rose to 26.2 per cent on the seventh day of treatment. His red cell count and hemoglobin values increased steadily even at this lowered level of administration. The patients who have been under treatment for from thirty-two to forty-one days have had erythrocyte increases from 1.05 million (Case 2) to 1.97 million per cubic millimeter (Case 9). One (Case 2) had a low-grade fever caused by abscessed teeth. Later in the study, dental extractions were performed, and her temperature thereafter was normal. Her erythrocyte count rose 1,000,000 per cubic millimeter in the following nine days. The hemoglobin values of all the cases increased as the erythrocyte count increased. The leucopenia generally accompanying macrocytic anemia was decreased by the administration of folic acid. In some cases the white counts rose as high as 20,000 per cubic millimeter and in other cases it increased to normal levels. The elevations did not persist, but white counts remained from 5,000 to 9,000 per cubic millimeter.

The normal control subjects who received 200 mg. of folic acid orally for ten days did not show any changes in their erythrocyte counts, hemoglobin, leucocyte counts, or number of reticulocytes in the four weeks following its administration.

In four of the patients who have been treated with folic acid for more than thirty days, the mean corpuscular volumes have decreased as follows: Case 1, from 122 to 94 cubic microns; Case 7, from 130 to 114; Case 8, from 119 to 94; and Case 9, from 145 to 109.

Bone marrow studies were repeated in three patients (Cases 1, 8, and 9) who had been treated for thirty-seven, thirty-one, and forty-one days, respectively. Macroscopically, there was a gross change from light red, thick, gelatinous marrow to deep red fluid containing discrete clumps of cells. Microscopically, the erythroblastic arrest was substituted by a normoblastic hyperplastic regeneration. There was a shift in ratio of white cells to nucleated red cells of from 5 to 2 to from 5 to 6 (Table II). The findings in the bone marrow from the other patients after treatment will be reported later since these subjects are still under treatment and the reticulocytes are still high.

The appetite improved rapidly soon after medication was started. Those who were under treatment for long periods of time (from thirty to forty days) have tremendous appetites and often request additional servings. In every instance, the glossitis has faded, and in five subjects (Cases 1, 2, 4, 5, and 9) the tongue has become entirely normal. The soreness of the tongue and mouth has totally disappeared, and patients can now eat and smoke without any sensation of burning of the tongue or mouth. The rectal burning about which some patients complained has also disappeared.

The paresthesias of the hands and feet have not yet been affected by the administration of folic acid. Edema disappeared in all patients with the exception of two (Cases 3 and 9). One subject (Case 3) had anasarca and nervous manifestations that might have been due to thiamine deficiency; she has been under treatment only fourteen days. Another subject (Case 9) had a reappearance of discrete ankle edema. At the time this report was submitted, the abdominal distention had not disappeared, and the patients still complained of "gas."

The diarrhea followed a variable course. It gradually decreased in most cases. At present, soft brown stools tending toward normal have replaced the original typical stools seen in sprue in four patients (Cases 3, 4, 5, and 6). The number of stools has decreased from three to fourteen to from one or two daily. At present the stools of one subject (Case 2) have returned to normal, but this patient had, in addition to folic acid, supplementary nicotinic acid therapy. Two subjects (Cases 8 and 9) still pass the same spruiform type of stools as they did when they were admitted, although for the last two days the stools of one of them (Case 8) have been soft and brown. The fact that there is such variation in the stools from normal to pathologic, and vice versa, leads us to the conclusion that at present no dogmatic statement can be made concerning the efficacy of folic acid alone in controlling the diarrheal aspect of sprue. Those subjects showing persistent improvement of the diarrhea with no tendency toward relapse to the spruiform type are the ones in whom the illness is of more recent onset. In these patients the course of therapy has been more effective in controlling the diarrhea than it has been in the chronic

cases. In all patients, shortly after the beginning of treatment, there was an accompanying physical improvement and sense of well-being. There was a tendency for the stools to become well formed, more normal in color, and much less in amount. For several days the stools would appear practically normal but this period would be followed for a day or so by copious, frothy stools. We are still studying the effect of folic acid on the alimentary tract and specifically upon the appearance of the stools. It suffices to say at this time that there is a great change in the majority of instances toward the normal following administration of folic acid.

DISCUSSION

The present findings show that synthetic folic acid produces great improvement in patients with tropical sprue in relapse. These, as well as previous observations by one of us (T. D. S.) and associates, demonstrate that folic acid has a striking antianemic effect in nutritional macrocytic anemia and sprue. Moore, Bierbaum, Welch, and Wright⁹ have confirmed these observations on the antianemic effect in pernicious anemia and have reported that folic acid has proved effective in one case of nontropical sprue and one case of pernicious anemia of pregnancy. Little is understood about the pathogenesis of these macrocytic anemias, and it is possible that these studies will open a new era in effecting a better understanding of the physiology of the blood-forming organs. Until more information is available, we suggest that patients who have achylia following histamine injection be considered tentatively to have Addisonian pernicious anemia and that, in cases with proved steatorrhea and macrocytic anemia, sprue be considered as a tentative diagnosis. Our criteria for the diagnosis of nutritional macrocytic anemia have been described fully in previous publications, and, after further intensive investigations, we see no reason to change our method of making this clinical diagnosis.⁵ We now suggest, however, that the so-called macrocytic anemia of pellagra and the pernicious anemia of pregnancy, which have been found to respond to folic acid, be considered as forms of nutritional macrocytic anemia and classified as such.

The physician must always make every effort to prove each diagnosis. Because it is often difficult to grasp the fundamental mechanism behind the disease, in many cases the clinical diagnosis is arbitrary. Certainly more studies are needed.

Despite the fact that pernicious anemia is included in the group of macrocytic anemias, it is obvious that in its natural pathogenesis it is somewhat different from the macrocytic anemia of sprue and from nutritional macrocytic anemia. One of us (T. D. S.) has the working hypothesis that folic acid in many of the foods occurs as a conjugate and that, in view of Castle's work, it is likely that in pernicious anemia the enzymes are unable to liberate the folic acid efficiently whereas in sprue, pellagra, pregnancy, and nutritional macrocytic anemia, the folic acid or substances acting similarly are more available.

Some explanation must be made concerning the fact that fairly large amounts of folic acid are required to produce a maximum hemopoietic response in contrast to potent liver extract in which the active substance in the thera-

peutic dose is apparently smaller. (One of us, T. D. S., has observed three patients who did not respond significantly on 5 mg. of folic acid but who did respond fairly satisfactorily on 10 mg. per day administered by mouth.) Unpublished observations by one of us (T. D. S.) show that a concentrate of dried brewer's yeast* produces a satisfactory hemopoietic response when 100 Gm. of the material is given by mouth daily. This amount of material contains approximately 1 mg. of folic acid. An explanation of this is probably similar to that previously described for the liver extract.

Since the clinical improvement that follows the administration of folic acid parallels that which follows the administration of potent liver extract, one might expect that folic acid in adequate doses would be as effective as liver extract in treating sprue. That folic acid is an effective hemopoietic agent is certain, but it probably will not have a curative effect above and beyond that afforded by large doses of a potent liver extract.

It seems likely that the natural pathogenesis of sprue and nutritional macrocytic anemia is related to an inadequate diet. We have found that animal protein or yeast and the nutrients included are effective in the treatment and prevention of this type of anemia. Yet, despite the fact that the patients in this study were restricted to a diet deficient in protein throughout the study, they had a striking clinical and hemopoietic improvement. (We recommend a high vitamin, high protein diet in the treatment of sprue, as folic acid cannot be expected to substitute for an adequate diet.)

Many of these patients were infested with intestinal parasites and they improved without any antiparasitic therapy, a point which will be more fully discussed in subsequent papers.

These studies on tropical sprue support the practical point of view that folic acid has a striking antianemic effect in some types of macrocytic anemias in relapse.

SUMMARY AND CONCLUSIONS

The present study has shown that synthetic folic acid produced great improvement in nine patients with tropical sprue in relapse. The subjects all had a good gain of body weight, they gained in strength, and the glossitis disappeared. The stools in all cases tended to become much nearer normal and in some instances they appeared normal. Studies directed toward obtaining a better understanding of the function of the alimentary tract are now under way. These studies and the previous ones by one of us (T. D. S.) and associates demonstrate conclusively that synthetic folic acid has a striking anti-anemic effect on persons with sprue, pernicious anemia, and nutritional macrocytic anemia and open a new era of better understanding of the physiology of blood formation. Despite the fact that pernicious anemia is included in the group of macrocytic anemias responding to folic acid therapeutically, it is obvious that its natural occurrence is different from that of sprue and of nutritional macrocytic anemia. Folic acid should be considered an antianemic substance, and our observations would suggest that the response to liver extract and

*Basamin, Anheuser-Busch, St. Louis, Mo.

to dried brewer's yeast powder is out of proportion to the amount of folic acid which they contain. It is truly remarkable that the patients improved so much from a clinical and laboratory point of view while restricted to a diet devoid of meat and meat products. We do not recommend such a restriction in diet in the day-to-day treatment of sprue. In fact, we stress a high vitamin, high protein diet as folic acid cannot be expected to substitute for an adequate diet.

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LABORATORY METHODS

A METHOD FOR DETERMINING THE RELATIVE ANTICONVULSIVE ACTIVITY OF BARBITURATES

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PREVIOUSLY one of us demonstrated the prolonged activity of pentobarbital and phenobarbital in antagonizing the convulsant action of either strychnine or picrotoxin.¹ It was suggested that this work might serve as the basis of a method for determining the comparative length of action of barbiturates. Although further studies indicated that this antagonism does not yield a true indication of the length of action of barbiturates relative to sedation or anesthesia, some information was obtained on the comparative protection offered by the barbiturates against convulsants and the duration of such protection. It was thought that this information might serve a useful function for some types of work. Although both picrotoxin and strychnine were used to produce death by convulsions, only picrotoxin proved satisfactory.

PROCEDURE

In these experiments male rats of a uniform strain (Purdue), source, and weight from (66 to 80 grams) were used, unless otherwise indicated. The animals were fed a commercial ration* ad libitum. At least ten rats were used for each run. When more than ten rats were used, it was to confirm a previous result. Since in each instance there was no significant difference, the data indicate that ten rats of this strain and of the same sex and weight were adequate. Rats from another source were tried in which ten rats of the same sex and weight were not adequate for consistent results.

All injections, 1 c.c. per 100 grams of body weight, were made intraperitoneally. Sodium salts of the barbiturates were used when available. When they were not available, the barbiturates were put into solution with the least possible amount of normal sodium hydroxide. The dose of strychnine sulfate was 3 mg. per kilogram. This dosage regularly killed seven of ten rats in the weight range used. The dose of picrotoxin was 10.5 mg. per kilogram. This dose killed nine of ten rats in the weight range of from 66 to 80 grams and seven of ten rats in the weight range of from 82 to 104 grams. The rats were weighed before the barbiturate was given and reweighed before the convulsant when the interval was longer than two hours. In such cases the second weight was always taken as the basis for the dose of convulsant. The temperature of the room where the experiments were done was kept at from 77 to 81° F. to rule out the effect of variations in temperature.² The rats were placed in this room at least twenty hours before an experiment was started.

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*Purina dog chow checkers.

A protective dose of barbiturate was determined. This was done by giving doses of barbiturate increased by increments of 20 per cent until not more than one of ten rats died from the convulsant given at the standard interval after the barbiturate. A twenty-minute standard was used for those compounds too short in action for a feasible dose of barbiturate to be given one hour before the convulsant. The number of deaths caused by the convulsant, when given at longer intervals after the barbiturate, was then compared with the number of deaths determined at the standard interval.

RESULTS AND DISCUSSION

As may be seen from Table I, several barbiturates required a fatal dose to be effective against strychnine. Of the nine compounds listed, only sodium phenobarbital did not produce any deaths at the dose level required. At a dosage greater than the L.D.₅₀, sodium ortal failed to protect the survivors against strychnine. Data obtained from these studies with strychnine are presented in Table II. With sandoptal, the protective action was gone in two hours. Sodium amytal, sodium pentobarbital, and sodium n-butyl allyl bar-

TABLE I. EFFECT OF SUFFICIENT BARBITURATE TO REDUCE DEATHS FROM STRYCHNINE SULFATE* TO 10 PER CENT OR LESS

BARBITURATE	DOSE (MG./KG.)	NUMBER OF RATS USED	NUMBER OF RATS DEAD FROM BARBITURATES	PER CENT OF RATS DEAD FROM BARBITURATES
Na phenobarbital	75	120	0	0
Na amytal	75	34	1	3
Neonal	62.5	45	4	9
Sandoptal	75	28	5	18
n-butyl allyl Na barbiturate	80	27	6	22
Na pentobarbital	40	26	6	23
Dial	100	79	29	37
Na barbital	300	213	84	39
Na alurate	90	67	32	48

*The dose of strychnine sulfate was 3 mg. per kilogram. This killed 70 per cent of untreated rats.

TABLE II. BARBITURATES LISTED IN ORDER OF INCREASING LENGTH OF ACTION AGAINST STRYCHNINE

BARBITURATE	DOSE (MG./KG.)	TIME AFTER BARBITURATE—3 MG./KG. STRYCHNINE SULFATE GIVEN							
		ONE HOUR		TWO HOURS		SIX HOURS		FORTY-EIGHT HOURS	
		NUM- BER OF	NUM- BER OF	NUM- BER OF	NUM- BER OF	NUM- BER OF	NUM- BER OF	NUM- BER OF	NUM- BER OF
		RATS USED	RATS DEAD	RATS USED	RATS DEAD	RATS USED	RATS DEAD	RATS USED	RATS DEAD
Sandoptal	75	10	0	10	9				
Na amytal	75	10	0	10	5				
Na pentobarbital	40	10	0	10	5				
n-butyl allyl Na barbiturate	80	10	0	10	5				
Dial	100	10	1	10	2	20	13	10	8
Neonal	62.5	10	0	10	2	10	3	10	9
Na alurate	90	10	0	10	1			10	4
Na barbital	300	10	1	10	0	10	1	30	11
Na phenobarbital	75	10	0	10	1	20	3	20	3

biturate exerted very little, if any, protective activity two hours after administration. Dial showed protection at two hours but not at six hours. Neon showed protection at six hours but not at forty-eight hours after its administration. Sodium alurate, sodium barbital, and sodium phenobarbital gave evidence of protection forty-eight hours after administration. The grouping of these compounds was much the same as that derived from other methods of determination of length of action.^{3, 4} However, strychnine as a test substance was abandoned because it (1) failed to give real additional information, (2) required too many animals, and (3) required the use of animals resistant to the barbiturate.

TABLE III. BARBITURATES SHOWING INTERMEDIATE AND PROLONGED PROTECTION AGAINST PICTROTOXIN

BARBITURATE	DOSE (MG./KG.)	TIME AFTER A BARBITURATE—10.5 MG./KG. OF PICTROTOXIN WAS GIVEN											
		ONE HOUR		TWO HOURS		FORTY- EIGHT HOURS		SEVENTY- TWO HOURS		NINETY- SIX HOURS		ONE HUNDRED TWENTY HOURS	
		NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD
Na ortal	110.0	10	1	10	8	10	6						
Na amytal	30.0	10	1	10	7	10	8						
Na pentobarbital	16.0	10	0	30	20	30	4	10	3	10	7		
Neonal	13.6	10	0	20	11	10	5	10	6				
Na alurate	30.0	10	1	10	3	10	6	10	6				
Na barbital	60.0	10	0	10	1	10	2	10	2	10	6	10	6
Na ipral	20.0	10	0	10	1	10	0	10	0	10	5	10	8
Na phenobarbital	37.5	10	1	10	1	10	0	10	1	10	3	10	8

When picrotoxin was used as the convulsant agent, the results indicated that supplemental information, not obtained by other methods commonly in use, was obtained. For convenience in discussion, the eight barbiturates whose standard protection against picrotoxin was determined at one hour were divided into two groups: (1) those in which picrotoxin at two hours killed more than 50 per cent, and (2) those in which picrotoxin killed less than 50 per cent in a similar period (Table III and Fig. 1). Of the four compounds which gave less than 50 per cent protection at two hours, the sodium salts of amytal and ortal gave results not significantly different from each other. Neon showed the same type of result, but the maximum death rate was not reached as quickly. Sodium pentobarbital showed a different type of result; whereas at two hours, 67 per cent were killed, at forty-eight hours only 13 per cent were killed. At seventy-two hours, 30 per cent were killed and at ninety-six hours 70 per cent were killed. The chance that the difference between the forty-eight hour value and the two-hour value was not significant was less than 1 in 100,000. The chance that the difference between the two-hour value and the seventy-two value was not significant was less than 1 in 100. Extra rats were run at these intervals as can be seen from Table III. There was no difference in results between the different runs. At present there is no explanation for this increase of protective action after the partial disappearance of protection.

Some studies on another group of rats indicated a similar type of protection, but the timing was different for the other strain of rats.

Of those compounds which showed less than 50 per cent deaths at two hours, the action of sodium alurate was the most brief. The protection seemed to be gone at forty-eight hours. There was no real difference between sodium barbital and sodium ipral. They both showed significant protection at seventy-

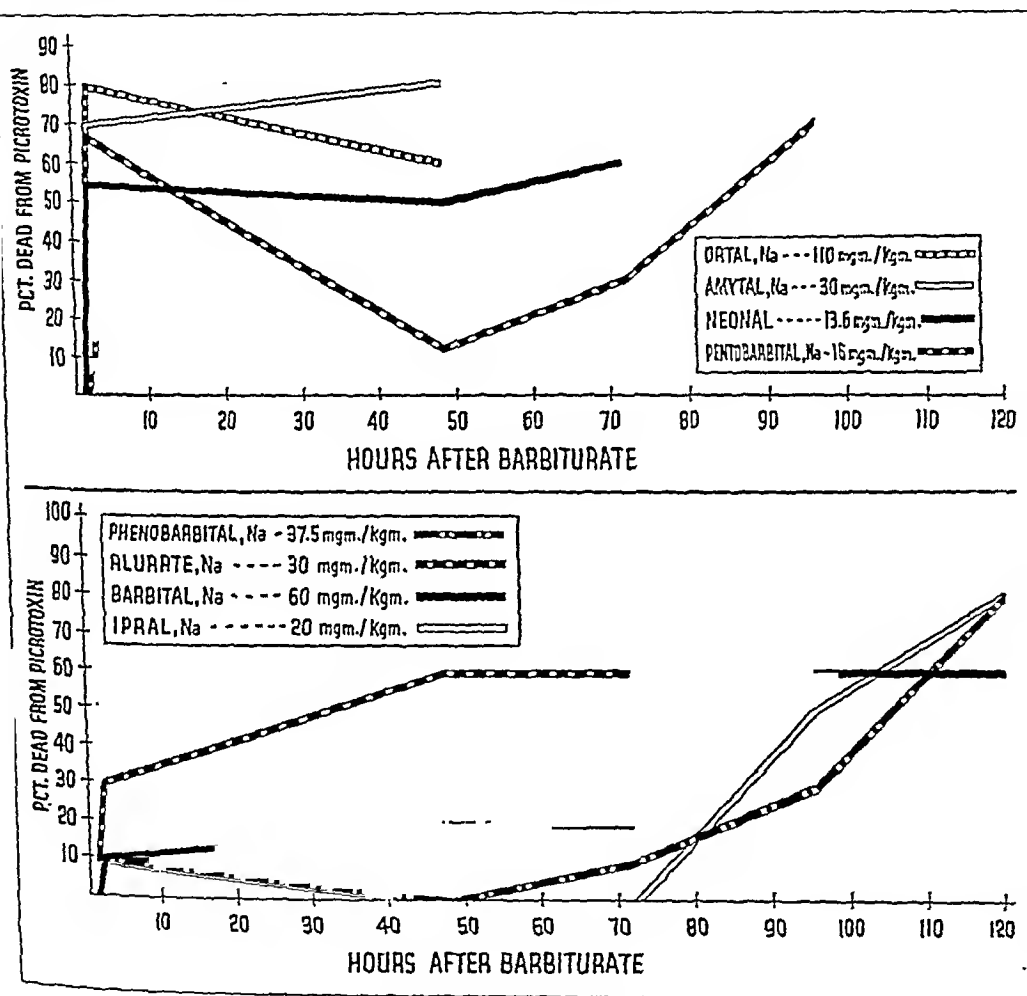


Fig. 1.—Deaths from picrotoxin after barbiturates with medium and long anticonvulsive activity.

two hours but not at ninety-six hours. Sodium phenobarbital apparently showed some protection at ninety-six hours, but at 120 hours the effect of all barbiturates was worn off. Therefore, it may be assumed that ninety-six hours would be sufficiently long to run the test for real differentiation. Of the eight compounds which were studied with the standard at one hour, sodium ortal was the only one which required an anesthetic dose. This dose

TABLE IV. BARBITURATES SHOWING SHORT PROTECTION AGAINST PICROTOXIN

BARBITURATE	DOSE (MG./KG.)	TIME AFTER A BARBITURATE—10.5 MG./KG. OF PICROTOXIN WAS GIVEN									
		TWENTY MINUTES		FORTY MINUTES		SIXTY MINUTES		EIGHTY MINUTES		TWO HOURS	
		NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD
Na seconal	18.0	10	0	10	5	10	10	10	10	10	10
Sandoptal	25.0	10	0	10	4	10	7	10	9	10	9
Na pentothal	15.0	30	2	30	8	30	13	30	6	30	20
Na ortal	90.0	10	1	10	0	10	3	10	7	10	10

killed about one in thirty rats. For this reason sodium ortal was also tested with a standard interval of twenty minutes.

For the shorter acting compounds, the standard was determined at twenty minutes after the barbiturates (Table IV and Fig. 2). The dose of sodium ortal for this interval was not lethal but was anesthetic. Sandoptal was tried in doses up to the dose used against strychnine at one hour. Since sandoptal was still not effective against picrotoxin, that compound was tested at twenty

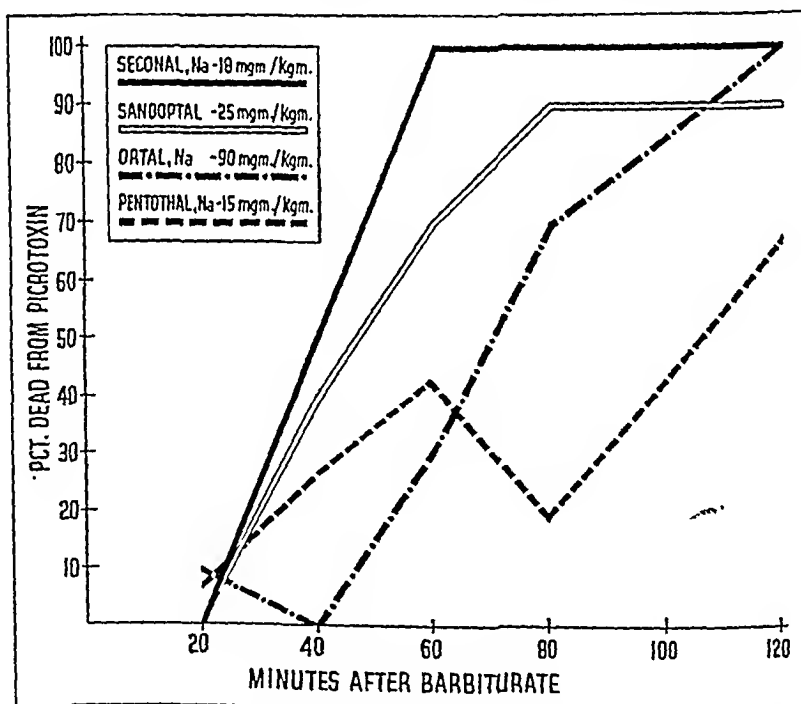


Fig. 2.—Deaths from picrotoxin after barbiturates with short anticonvulsive activity.

minutes. Here a dose just approaching the anesthetic level was necessary. Sandoptal was the only barbiturate in which the protective dose against picrotoxin was not smaller than the protective dose against strychnine for the one-hour standard. It can be seen that the protective action of sandoptal

was very short. It might be that since picrotoxin took longer to produce convulsions and kill than strychnine, most of the activity was gone by the time picrotoxin became effective; whereas, that was not true of strychnine. Of the four compounds studied with a twenty-minute standard for picrotoxin, sodium seconal, sandoptal, and sodium oral all showed consistent decrease in activity with time. The activity of sodium seconal decreased most rapidly and that of sodium oral least rapidly. Sodium pentothal showed a consistent decrease through the sixty-minute interval and then apparently an increase in activity at eighty minutes. The chance that the difference between these two times was not significant is about 1 in 20. It would appear that pentothal showed the same phenomenon as pentobarbital but not in so striking a manner.

SUMMARY

Strychnine proved to be unsatisfactory for testing the comparative anticonvulsant action of barbiturates. Picrotoxin was a satisfactory agent for measuring this action. Ten male rats of the strain used weighing from 66 to 80 grams inclusive proved to be a sufficient number to give consistent results. The barbiturates with short duration of anticonvulsive activity were studied with a standard interval determined at twenty minutes after the administration of the barbiturate. Those with medium and long duration of anticonvulsive activity were studied with a standard determined at one hour. Loss of anticonvulsive activity was usually continuous and varied from an hour to four days for complete loss. With two compounds studied there was an early loss of activity followed by a partial regaining of anticonvulsive activity before the final loss of activity. The information obtained did not replace information obtained by other methods. It is believed that this method might give supplemental information which would be useful in some instances.

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TABLE IV. BARBITURATES SHOWING SHORT PROTECTION AGAINST PICROTOXIN

BARBITURATE	DOSE (MG./KG.)	TIME AFTER A BARBITURATE—10.5 MG./KG. OF PICROTOXIN WAS GIVEN									
		TWENTY MINUTES		FORTY MINUTES		SIXTY MINUTES		EIGHTY MINUTES		TWO HOURS	
		NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD	NUM- BER RATS USED	NUM- BER RATS DEAD
Na seconal	18.0	10	0	10	5	10	10	10	10	10	10
Sandoptal	25.0	10	0	10	4	10	7	10	9	10	9
Na pentothal	15.0	30	2	30	8	30	13	30	6	30	20
Na ortal	90.0	10	1	10	0	10	3	10	7	10	10

killed about one in thirty rats. For this reason sodium ortal was also tested with a standard interval of twenty minutes.

For the shorter acting compounds, the standard was determined at twenty minutes after the barbiturates (Table IV and Fig. 2). The dose of sodium ortal for this interval was not lethal but was anesthetic. Sandoptal was tried in doses up to the dose used against strychnine at one hour. Since sandoptal was still not effective against picrotoxin, that compound was tested at twenty

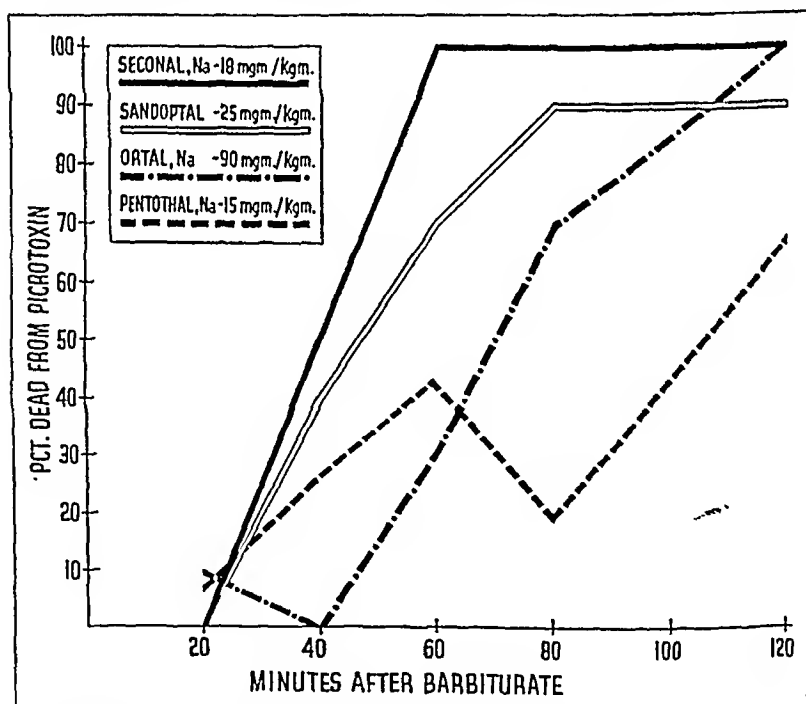


Fig. 2.—Deaths from picrotoxin after barbiturates with short anticonvulsive activity.

minutes. Here a dose just approaching the anesthetic level was necessary. Sandoptal was the only barbiturate in which the protective dose against picrotoxin was not smaller than the protective dose against strychnine for the one-hour standard. It can be seen that the protective action of sandoptal

was very short. It might be that since pierotoxin took longer to produce convulsions and kill than strychnine, most of the activity was gone by the time pierotoxin became effective; whereas, that was not true of strychnine. Of the four compounds studied with a twenty-minute standard for pierotoxin, sodium seconal, sandoptal, and sodium ortal all showed consistent decrease in activity with time. The activity of sodium seconal decreased most rapidly and that of sodium ortal least rapidly. Sodium pentothal showed a consistent decrease through the sixty-minute interval and then apparently an increase in activity at eighty minutes. The chance that the difference between these two times was not significant is about 1 in 20. It would appear that pentothal showed the same phenomenon as pentobarbital but not in so striking a manner.

SUMMARY

Strychnine proved to be unsatisfactory for testing the comparative anti-convulsant action of barbiturates. Pierotoxin was a satisfactory agent for measuring this action. Ten male rats of the strain used weighing from 66 to 80 grams inclusive proved to be a sufficient number to give consistent results. The barbiturates with short duration of anticonvulsive activity were studied with a standard interval determined at twenty minutes after the administration of the barbiturate. Those with medium and long duration of anticonvulsive activity were studied with a standard determined at one hour. Loss of anticonvulsive activity was usually continuous and varied from an hour to four days for complete loss. With two compounds studied there was an early loss of activity followed by a partial regaining of anticonvulsive activity before the final loss of activity. The information obtained did not replace information obtained by other methods. It is believed that this method might give supplemental information which would be useful in some instances.

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AN IN VITRO METHOD FOR DETERMINING THE RESISTANCE OF BETA HEMOLYTIC STREPTOCOCCI TO SULFADIAZINE*

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THE appearance of a number of strains of Group A beta hemolytic streptococci which are highly resistant to sulfadiazine makes their recognition by in vitro tests highly desirable. Certain in vitro procedures used heretofore for this purpose have been somewhat cumbersome for routine clinical use. We have been able to differentiate between common strains of group A streptococci which are susceptible to sulfadiazine and certain highly resistant epidemic strains by a relatively simple method. The concentration of sodium sulfadiazine required to cause a 50 per cent slowing of the growth rate of the organisms is measured; a casein hydrolyzate, beef infusion medium enriched with small amounts of serum is used.

Similar methods have been used extensively in antibiotic studies. Kohn and Harris¹ have shown that such a method is well adapted for determining the sulfonamide resistance of bacteria in media containing sulfonamide antagonists. They have carried out exhaustive studies of growth rates of bacteria in media containing sulfonamides and various sulfonamide antagonists¹⁻³ and have shown that in such a medium the influence of sulfonamides on bacterial growth rates is constant only if an appropriate degree of growth inhibition is measured.^{1,4} The measurement of a degree of inhibition below a certain critical level minimizes the influence both of the para-aminobenzoic acid³ type of antagonist and of secondary types.¹

The rationale for methods of the type proposed is discussed by Kohn⁴ in a consideration of the principles involved in designing a method for study of sulfonamide antagonists and synergists. He states that the type of method advocated can be adapted for determining sulfonamide resistance of bacteria in vitro. The desirability of using a growth rate method to compensate for cultural differences of different strains of bacteria is stressed. The fallacy of reading all tests at a definite time is pointed out. The leeway afforded in the size of the inoculum and other advantages of this type of test are discussed.

PROCEDURE

Preparation of Medium.—Fat and papillary muscle are removed from fresh beef heart. The heart is ground and infused overnight in a refrigerator in 1,000 c.c. distilled water for each pound of ground heart. It is then heated and a temperature of 85° C. is maintained for thirty minutes. The fat is skimmed from the surface and the suspension is filtered through gauze and coarse filter paper. The filtrate is made up to volume with distilled water.

*Army Air Force Rheumatic Fever Control Program.
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To each 800 c.c. of the infusion, 200 c.c. vitamin-free casein hydrolyzate* in 10 per cent solution are added. The mixture is warmed to room temperature or above, and to each 1,000 c.c. the following chemicals are added:

Sodium chloride	2.0 Gm.
Dextrose	2.0 Gm.
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$	1.0 Gm.

The pH is adjusted to 7.6 and the material brought to boiling. It is then filtered and the pH is again checked.

From 16 to 18 c.c. are distributed in Nessler type pyrex tubes and autoclaved at 121° C. for fifteen minutes. The final pH will be from 7.3 to 7.4. Values below this and above 7.8 are unsatisfactory.

The medium may be stored in the refrigerator until it is needed, at which time 1 per cent sterile rabbit, human, or horse serum is added. The serum need not be inactivated in the water bath.

Preparation of Sulfadiazine Tubes.—A solution of sodium sulfadiazine in N/100 NaOH, which contains 1,280 mg. sulfadiazine per 100 c.c., is prepared. By appropriate dilutions with N/100 NaOH, solutions of 10, 40, 160, 320, and 640 mg. per cent sulfadiazine are also prepared.

Each test requires seven Wassermann type test tubes. One contains 0.25 c.c. distilled water; the other six contain, respectively, 0.25 c.c. of 10, 40, 160, 320, 640, and 1,280 mg. per cent sodium sulfadiazine. These tubes are plugged and sterilized in an Arnold sterilizer on two successive days for an hour. They may be stored indefinitely if evaporation is prevented.

Equipment Necessary for Each Test.—

1. From 16 to 18 c.c. sterile casein broth.
2. Kahn type test tube containing 0.5 c.c. sterile casein broth.
3. Fifteen- to eighteen-hour culture of test organism in casein broth.
4. Control and six sulfadiazine tubes (see under Preparation of Sulfadiazine Tubes).
5. Sterile plugged graduated 10 c.c. pipette.

Procedure.—Between 8 and 10 A.M. an eighteen-hour culture of the organism is agitated several times and a loopful (platinum loop of from 2 to 3 mm. inside diameter) is transferred to 0.5 c.c. warm casein broth. (It is desirable to keep all broth as near 37° C. as is practical.) After agitation, a loopful of material is transferred from the 0.5 c.c. tube to a tube containing 18 c.c. of warm broth.

This 18 c.c. tube of inoculated broth is mixed thoroughly either by being aspirated with bulb and pipette ten times or by being placed for five minutes in a Kahn shaker. Then 2.25 c.c. of the inoculated broth are transferred to each of the control and six sulfadiazine tubes. The final sodium sulfadiazine concentration of each tube will be 0, 1, 4, 16, 32, 64, and 128 mg. per cent.

All control tubes are placed in one rack and the sulfadiazine tubes in another rack on the same shelf of the incubator at from 36 to 37° C. It is most important that the temperature is not allowed to go above 37° C.⁵

*General Biochemicals, Inc., Chagrin Falls, Ohio.

The rack of control tubes is inspected during the evening and the time at which each becomes visibly turbid is noted. For most clinical work, it is sufficient to inspect the tubes at about 8 and 11 P.M.

Reading and Reporting Tests.—The sulfadiazine tubes are read when they have incubated for approximately twice as long as the time required for the control tube to become turbid. It is assumed that if an inoculum requires twice as long to achieve visible turbidity in a sulfonamide as in a control tube, the growth rate in the former was only half that of the control. We have adopted the following routine: If the control tube was turbid by 8:00 P.M., the corresponding sulfonamide tubes were examined at from 8:00 to 9:00 A.M. At

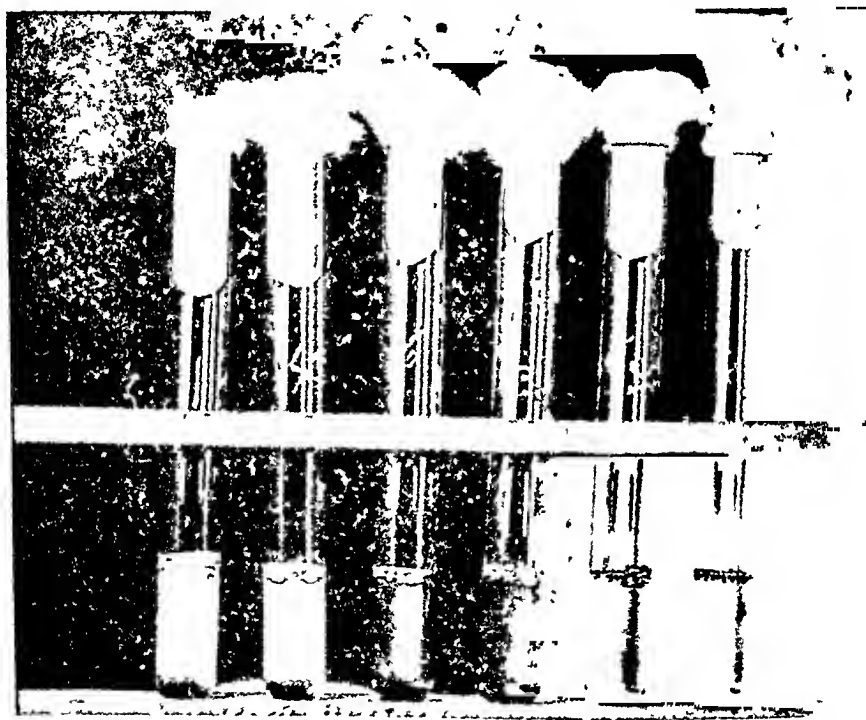


Fig. 1.—Sulfadiazine-susceptible strain of Group A beta hemolytic streptococcus showing end point at 4 mg. % sulfadiazine. Many strains will show no visible growth in any sulfonamide tube (Official photograph, United States Army Air Forces.)

this time all strains which had grown out in media containing as much as 128 mg. per cent sulfadiazine were tentatively reported "resistant," pending culture for purity of the highest sulfonamide tube showing growth. Sulfonamide tubes for which the control tube became turbid by 11:00 P.M. were read at noon. The few sulfonamide tubes for which the corresponding control became turbid after 11:00 P.M. and before the following morning were arbitrarily read late the following afternoon.

Before the six sulfonamide tubes are examined, they should be shaken vigorously for a few seconds. It is usually noted that all tubes below a certain sulfadiazine concentration will show a heavy turbidity, while those above that level will be clear (Fig. 1).

The highest sulfonamide-containing tube showing definite turbidity is regarded as the concentration of sodium sulfadiazine causing 50 per cent inhibition of the growth rate and is so reported. Tubes in which the clump of organisms is so small as not to be obvious after shaking are regarded as clear. Rarely the end point is not definite, but an error of one tube does not usually influence the interpretation of results.

Known sulfadiazine-susceptible and -resistant strains are tested routinely (Figs. 1 and 2).

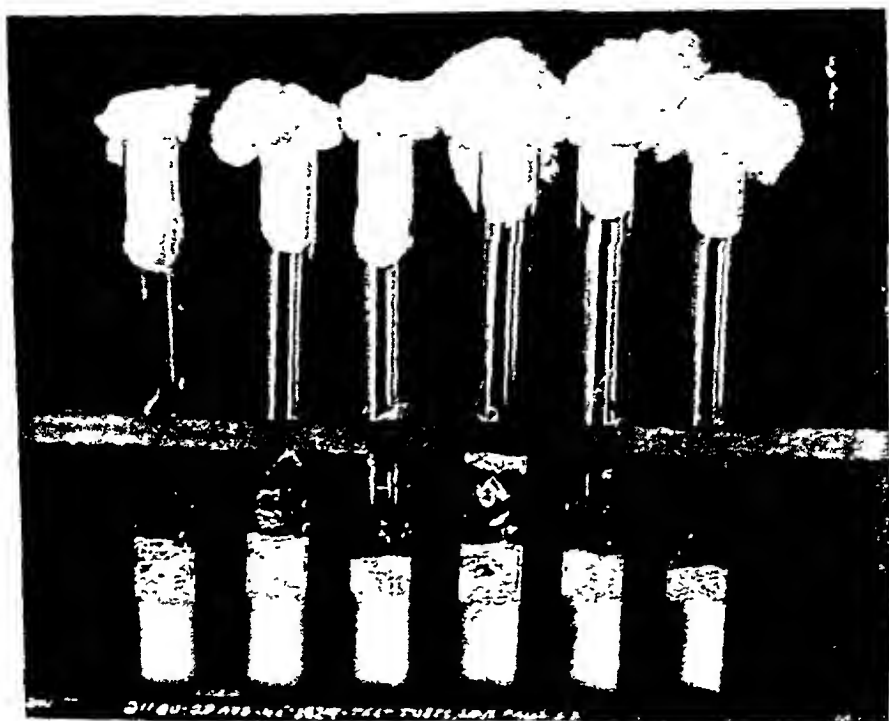


Fig. 2.—Sulfadiazine-resistant strain of Group A type 17 beta hemolytic streptococcus showing growth in all sulfadiazine tubes from 1 to 128 mg. per cent sulfadiazine. (Official photograph, United States Army Air Forces.)

RESULTS OBTAINED

Group A streptococci from the Denver area and a number of Air Force installations throughout the country were tested at Buckley Field, Colorado, by the proposed method. The clinical response to sulfadiazine of a number of the strains tested was known. An epidemic of streptococcal infections caused by a highly sulfadiazine-resistant strain of type 17 streptococcus (reported elsewhere) furnished a large number of clinical cases with which to check in vitro results. This limited experience indicated that organisms would be susceptible to therapeutic administration of sulfadiazine if they grew only in 4 mg. per cent concentration of sulfadiazine or less and resistant to therapy if they were able to grow in concentrations of 64 mg. per cent or more. Intermediate values for in vitro resistance gave less satisfactory clinical correlation. The parallel between clinical response and in vitro resistance with most strains of Group A

streptococci was close because most strains encountered by us were either markedly sulfadiazine-susceptible or -resistant.

Certain strains of streptococci of varying degrees of sulfadiazine resistance were tested from thirty to fifty times in different batches of medium. Variation in results of more than one tube occurred infrequently. The test is therefore sufficiently accurate to be of clinical value.

The Wilson⁶ method of determining sulfadiazine resistance of streptococci in vitro has been used by Shott⁷ at another laboratory. A series of 318 Group A streptococci of different types were tested by the proposed method and the Wilson method. Radical differences between the two methods prevent direct comparison of sulfonamide values obtained. There was agreement on the in vitro resistance to sulfadiazine for 94 per cent of the strains tested. It is of interest that both laboratories working independently were in agreement that streptococci of types 3, 17, 18, 19, and 30 are sometimes sulfadiazine resistant in vitro. Shott⁷ also reported that strains of types 1 and 14 were resistant in vitro, these types having been encountered infrequently by us. No Group A streptococci other than strains of the five types mentioned were found resistant by the proposed method.

SUMMARY

1. A simple method of determining the sulfadiazine resistance of Group A beta hemolytic streptococci is described.

2. The test is based on the observation of Kohn and Harris, that sulfonamide antagonists do not influence results greatly if an appropriate degree of growth inhibition is measured.

The author is greatly indebted to Pfc. H. L. Kohn for instructions in designing this test.

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EFFECT OF METALS ON GIEMSA STAIN SOLUTIONS IN FIFTY PER CENT GLYCEROL AND METHANOL MIXTURE

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BETHESDA, MD.

DURING World War II, one batch of Giemsa stain solution prepared for the use of the Naval Medical Corps deteriorated in service at various stations. Officers complained that all eosin staining was lost. This lot had given satisfactory staining and spectroscopic tests before issuance, and retained samples in the Naval Medical School and in the National Institute of Health still gave satisfactory tests.

Examination of spectroscopic data revealed a marked proportionate decrease in optical density at the eosin peak of from 516 to 518 $m\mu$ in the case of the two samples giving unsatisfactory stains. Heating the solutions and shaking them frequently for several days failed to remedy this defect. It was thought that exposure to outdoor winter temperatures might have occasioned precipitation of eosinates, thus decreasing the relative quantity of eosin remaining in solution, since Giemsa stain normally contains an added excess of thiazine dyes over the neutral eosinates.

At this point in the investigation, O. C. Western, Lt., and E. E. Ozburn, Lt. (j.g.), both M.C., U.S.N.R., in the chemistry laboratory of the Naval Medical Center, observed a yellowish incrustation on the tin-foil seals lining the screw caps of the Giemsa stain bottles and found that when this material was washed off in water, a yellowish pink solution was obtained.

Following these observations, we obtained some of the incrustated tin-foil cap liners and extracted them with aqueous sodium carbonate solution. These yielded red solutions with a yellow fluorescence on dilution. Spectroscopically, after dilution with water, they gave maximum absorption at λ 516; the 90 per cent density band was 11 and 12 $m\mu$ wide and medians were found to be 515.2 and 516.3. These data lie within the range given by usual samples of eosin Y in distilled water.

Next, some uncorroded tin-foil cap liners were immersed in laboratory samples of Giemsa stain at 37° C. When a 1:500 dilution in distilled water was examined spectroscopically, this stock stain gave a ratio of eosin density to thiazine density (DE/θ) of 0.571. In sixteen hours, this value reached 0.486; in seven days, 0.199. Since, in accordance with the Beer-Lambert law, optical density is directly proportional to the concentration of the solution, this fall in density at the eosin peak, in relation to the density at the thiazine peak, indicates a considerable loss of eosin from the stock solution. This experimentally altered sample presented the same loss of eosin staining as was observed in the deteriorated samples returned for re-examination.

From the Pathology Laboratory, National Institute of Health.

Received for publication, Nov. 14, 1945.

*Medical Director, U. S. Public Health Service.

TABLE I. SPECTROSCOPIC DATA ON VARIOUS SAMPLES OF ONE LOT OF GIEMSA STAIN

DESCRIPTION OF SAMPLES	DATE	EOSIN			THIAZINE COMPONENT			
		λ	D	R	λ	D	W	M
Original pre-issue examination	8/31/44	518	.264	.574	660	.460	21.5	636.6
Re-examination of above sample	2/20/45	516	.170	.555	660	.305	25.4	654.8
Same, heated	2/27/45	516	.202	.525	655	.385	24.9	653.4
Returned unsatisfactory sample	2/20/45	516	.100	.372	657.5	.270	25.4	654.5
Same, heated	2/27/45	516	.125	.352	655	.355	24.7	653.4
First sample from Naval Medical School Stores	2/20/45	518	.082	.246	657.5	.332	23.3	655.1
Same, heated	2/27/45	516	.090	.262	655	.345	24.9	653.8
Second sample from Naval Medical School Stores	2/20/45	518	.214	.560	657.5	.382	25.4	654.8
Same, heated	2/27/45	516	.221	.525	655	.421	23.2	654.3

λ = Absorption maximum.

D = Optical density at absorption maximum.

R = D at eosin absorption maximum divided by D at thiazine absorption maximum

W = Width of absorption band in which D exceeds 90 per cent of its maximum.

M = Median of absorption band in which D exceeds 90 per cent of its maximum.

The composition of the tin-foil* liners employed was unknown to us at that time. Therefore, we placed small quantities of various pure metals in 10 c.c. quantities of 0.8 per cent Giemsa stain in equal volumes of glycerol and methanol and incubated these at 37° C. In addition controls were used with no added material and with cardboard, cork, and rubber.

In the five controls, the ratio $DE/D\theta$ remained above .500 except for the forty-four day cardboard sample; it became .415. Similar behavior was noted with cobalt, chromium, lead, copper, silver, and one sample of brass. With calcium and magnesium there was a pronounced shift of the thiazine absorption band from the initial 658.2 median to 605.9 and 620.9 for the first two, respectively; with two samples of cadmium, the shift was to 638.5 and 616.5. This is interpreted as the usual effect of alkali.† With calcium, the $DE/D\theta$ ratio rose to 1.32 and 1.062 at fourteen and twenty-eight days, respectively; with magnesium, it remained in the usual zone; while with cadmium it fell in twenty-eight days to .323 for the first and to .290 for the second sample. With the second sample of brass, two samples each of aluminum, zinc, tin, arsenic, and antimony, the $DE/D\theta$ reached values below 0.300 in from fourteen to twenty-eight days or longer; with aluminum, sixty-three days were required and with arsenic, seventy-seven days (one sample each). The lowest values for the $DE/D\theta$ ratio were reached with zinc (.162, .129) and aluminum (.186, .172).

The two most active metals in the electrolytic series produced primarily alkali effects, probably because of their rapid conversion to hydroxides, and there was little or no adsorption of eosin. The lower metals in the electrolytic series apparently did not decompose the eosinate, while the middle members actively decomposed the eosinates and removed the eosin from solution.

With the exception of the calcium, cadmium, and magnesium samples noted, the thiazine absorption band median either remained fairly stationary between 658 and 650 or shifted moderately to the left (into the 640's); with one sample

*The composition of the tin foil used for these cap liners was furnished us from the manufacturer: tin 99.7 per cent; lead, not over 0.12 per cent; copper, 0.03 per cent; arsenic, 0.103 per cent; antimony, 0.08 per cent; and iron, not over 0.04 per cent.

†Lillie, R. D.: The Deterioration of Romanowsky Stain Solutions in Various Organic Solvents, Pub. Health Rep., No. 178 (Suppl.), 1944.

TABLE II. EFFECT OF INCUBATION OF GEMS STAIN AT 37° C. WITH VARIOUS METALS ON ABSORPTION SPECTRUM

	DAYS TIME	λ E	λ O	90% D θ		DE D θ
				W	M	
Control	0	516	660	27.9	658.2	0.655
	14	516	652.5	24.9	652.2	.769
	28	516	652.5	30.1	647.6	.765
Second Control	14	516	657.5	25.6	652.5	.711
	28	516	652.5	26.4	650.7	.547
Cardboard Control	14	518	652.5	19.3	650.8	.565
	44	517	650	28.6	647.8	.415
Cork Control	14	516	652.5	31.7	649.6	.555
	44	517	650	28.8	648.1	.619
Rubber Control	14	516	657.5	28.2	649.6	.619
	44	516	649	31.0	646.0	.730
Tin						
First sample	14	520	657.5	26.0	656.2	.249
Second sample	14	516	655	26.9	652.4	.270
	28	516	657.5	21.1	656.5	.250
Cadmium						
First sample	14	516	652.5	36.3	643.6	.475
Second sample	28	518	645	35.1	638.4	.323
	77	518	640	30.6	636.5	.243
	14	517	632.5	39.3	631.5	.320
	28	524	617.5	35.4	616.5	.290
Zinc						
First sample	13	518	660	26.5	661.0	.162
Second sample	14	504	655	23.6	654.1	.151
	28	498	659	20.6	657.4	.129
Aluminum						
First sample	13	521	660	31.6	652.4	.482
Second sample	63	516	645	33.4	640.0	.186
	14	516	651	26.4	648.5	.274
	28	519	650	20.0	650.0	.172
Magnesium	14	516	640	40.4	632.5	.859
	28	515	622.5	33.9	620.9	.683
Calcium	14	516	610	31.5	607.3	1.320
	28	514	603	30.1	605.9	1.062
Iron	14	520	657.5	26.5	655.0	.547
	63	518	650	31.0	646.2	.387
Cobalt	14	516	652.5	32.3	648.2	.779
	28	517	647.5	31.9	643.5	.699
Chromium	14	516	657.5	25.6	653.6	.730
	28	517	652.5	19.9	652.7	.677
Lead	14	520	660	26.9	657.4	.705
	63	516	650	35.5	643.1	.746
Copper	14	518	660	28.2	656.2	.673
	63	514	652.5	32.8	647.5	.614
Silver	14	517	657.5	25.2	655.3	.750
	28	514	657.5	23.4	654.6	.520
Brass						
First sample	14	516	660	27.2	654.1	.750
Second sample	28	516	650	25.9	650.4	.539
	14	515	646	28.8	645.0	.384
	28	516	649	29.3	646.0	.200
Arsenic	14	516	655	27.5	653.8	.545
First sample	28	516	655	24.6	652.6	.338
Second sample	77	516	647.5	27.7	649.0	.209
	14	517	650	32.5	643.4	.625
	28	522	645	34.4	634.5	.206
Antimony	14	516	662.5	24.6	654.3	.296
First sample	28	514	656	25.6	658.1	.228
Second sample	77	516	656	24.7	654.0	.299
	14	516	657.5	24.7	652.8	.246
	28	512	660	23.0	654.5	.209

 λ = Absorption maximum.

E = Eosin.

O = Thiazine.

D = Optical density at absorption maximum.

W = Width of absorption band in which Density (D) is over 90 per cent of its maximum.

M = Median of absorption band in which Density (D) is over 90 per cent of its maximum.

with arsenic, to 634.5. With zinc, however, the absorption band median shifted to 661 with one sample at thirteen days, and from 654 at fourteen days to 657.4 at twenty-eight days with the other.

In further exploration of this atypical shift, samples of thionin eosinate and of azure A eosinate¹ in 1 per cent solutions in glycerol methanol 50:50 volume mixture were incubated at 37° C. with metallic zinc. The shift of the ratio $DE/D\theta$ occurred as before, going from 1.000 to 0.399 in twenty-eight days with thionin eosinate and from .593 to .215 in two months with the azure eosinate. The thionin absorption band median remained constant in position between 595 and 599, both with and without zinc. The azure A absorption band median shifted from 622.9 to 638.0 in two months with zinc but remained between 621 and 623 without it.

The exact explanation of this phenomenon remains obscure. Formation of ethyl thionins has been suggested.

CONCLUSION

Various metals have the property of decomposing thiazine eosinates in glycerol methanol solution and removing the eosin from those solutions. These are relatively active metals in the electrolytic series. However, alkali earth metals appear to act purely as alkalies.

Zinc acts on methyl thionins in glycerol methanol solution, but not on thionin, to shift their spectral absorption bands toward the red end of the visual spectrum.

The use of tin foil or other foils containing tin, zinc, or cadmium for lining screw caps for Giemsa stain is contraindicated. Copper foils containing appreciable admixtures of zinc or tin seem to be similarly contraindicated. Paraffined cardboard seems to be the most innocuous and generally satisfactory lining material for such caps.

Acknowledgment is due to D. Plotka, for reading the absorption spectra and doing part of the mathematical calculation of derived spectroscopic data as well as for other technical assistance.

BOOK REVIEWS

Hematology for Students and Practitioners. By Willis M. Fowler, A.B., M.D., Professor of Internal Medicine, University of Iowa, Iowa City, Iowa: with a chapter by Elmer L. DeGowin, A.B., M.D., Assistant Professor of Internal Medicine, University of Iowa, Iowa City, Iowa. Paul B. Hoeber, Inc., New York, 1945. Price \$8.00. Cloth with 499 pages.

In this new volume on clinical hematology, Dr. Fowler presents, from the standpoint of the internist, the hematological viewpoint which he has exemplified in his own practice, teaching, and research at the University of Iowa in recent years. "Because of the wide variety of lesions which produce alterations in the circulating blood, hematology cannot be too definitely separated from the parent field of internal medicine, and diagnosis of diseases of the blood should be based on a consideration of the patient as a whole, not on examination of the blood alone." (Reviewer's italics.)

To the undergraduate medical student and the modern-day, intelligent practitioner of medicine, the current "theories" underlying the etiology and therapy of human disease are usually considered to be less essential than to the active investigator. On this assumption, sufficient background, devoid of detailed discussions, and adequate illustrative plates, both colored and black and white, with pertinent tables and graphs are provided to support the text and to illuminate the practical instructions given and conclusions drawn. With the same objectives in mind, the bibliographic references are brief and to the point, are seldom interjected into the text, and reflect the critical selection and judgment of the author.

The discussion of the anemias is excellent, including a timely chapter on the anemia secondary to blood donations, which provides the backdrop for Elmer DeGowin's thorough and authoritative discussion of all that has been learned in World War II about blood transfusion and the therapeutic use of blood derivatives. The present reviewer has yet to see anywhere so succinct and complete a résumé of this most important subject, which is of utmost significance to every physician-surgeon, internist, and medical specialist alike, of whatever particular interest. The basis for donor selection, the technics of collection, preservation, and typing of blood, including the Rh factor, the organization of a blood bank, the indications and contraindications for the use of blood and/or blood derivatives and substitutes, and posttransfusion reactions, their cause, prevention, and treatment, are all treated from the most practical clinical standpoint.

The differential diagnostic approach to the hemorrhagic and purpuric syndromes is clearly presented, and a very real service is performed in bringing some order out of a considerable degree of chaos in the thinking of many medical students and practicing physicians in these areas.

In contrast to the primary blood dyscrasias appears a brief chapter on the blood picture in a miscellaneous group of infections, which will be helpful to the general clinician.

An innovation worthy of emulation and elaboration is the separate chapter in this work devoted to a special consideration of the blood picture in infancy and childhood. Diagnostic and prognostic interpretations are decidedly conditioned by the age of the patient, and a recognition of this will avoid many errors in clinical judgment and therapeutic advice.

The volume is closed with a short chapter on routine laboratory procedures and an index facilitating rapid reference to specific detailed information. This volume reflects the improvement in format and quality of paper stock which the postwar period will now make possible.

Dr. Fowler has admirably accomplished his stated objectives.

CHARLES A. DOAN.

Pulmonary Edema and Inflammation. Harvard University Monograph in Medicine and Public Health No. 7. By Cecil K. Drinker, M.D., D.Sc., Professor of Physiology, School of Public Health, Harvard University, Boston, Mass. Harvard University Press, Cambridge, Mass. Price \$2.50. Cloth with 106 pages.

This small volume constitutes a series of four lectures delivered at the Bowman Gray School of Medicine in December, 1944, together with a fifth chapter on artificial respiration. Although the title of the book is perhaps somewhat misleading, since only the briefest mention is made of inflammation, the text is packed with valuable information and is written in a pleasant informal style punctuated by frequent touches of delightful humor.

The first lecture, dealing with structure, describes the vast capillary network of the lung and defines its relation to the more than 750 billion alveoli. The structure and function of the pulmonary lymphatics are authoritatively discussed and there is included a detailed description of the author's own method of cannulating the right lymphatic duct of the dog. Since in most dogs practically all of the lymphatics from both lungs drain into the right duct, it is possible to study pulmonary lymphatic flow by means of cannulating this delicate vessel.

In discussing the histology of the lung, Drinker quotes widely from the writings of William Snow Miller whose volume on the anatomy of the lung has for many years been accepted as the most authoritative treatise on the subject. It is of interest, therefore, that the author accepts without reservation the evidence presented by Loosli concerning the existence of the interalveolar communications known as the pores of Cohn. Miller, who contended that each alveolus was lined with a very thin layer of flat epithelium, claimed that these minute communications between the alveoli could be found only in pathological lung tissue and were absent in the normal lung. To refute this earlier claim, Drinker quotes extensively from Loosli's experiments and even goes so far as to publish two of his camera lucida drawings illustrating the pores of Cohn. The acceptance of this evidence is of particular significance since Drinker fails to mention the equally conclusive experiments of the same author disproving Miller's contention that each alveolus is lined with epithelium. Loosli has clearly demonstrated that the alveolar walls are made up only of capillaries supported in a network of reticulum and he has pointed out that the pores of Cohn merely represent the holes which normally exist in this network. To the reviewer at least, it appears illogical to accept the existence of the pores of Cohn and at the same time contend that the alveoli are lined with epithelium. Drinker not only ignores the important observations of Loosli regarding the structure of the alveolar walls, but he also makes frequent reference to the epithelial lining of the alveoli in interpreting the consequences of pulmonary edema.

The second lecture discusses physiological factors relating to pulmonary edema. At the outset, the author clearly states his belief that: "Simple pulmonary edema and the more serious pulmonary exudation depend more upon alterations in the permeability of the lung capillaries than upon complicated pressure relations in the pulmonary circulation." He presents considerable experimental evidence to support this view and emphasizes the role of anoxia in causing increased capillary permeability. Although the author's reasoning appears to be entirely sound, most of the evidence presented is indirect, and the reader is left with the impression that more direct methods of experimentation must be employed before the statement can be conclusively proved. Of greatest interest in this lecture is the discussion of an unnamed derivative of thiourea which apparently exerts a selective action upon the pulmonary capillaries, causing little, if any, change in the other capillaries of the body. The drug, which is extremely insoluble in water, when injected intravenously, causes profound pulmonary edema. The fluid leaking from the pulmonary capillaries is at first carried off by the lymphatic drainage, but eventually the leakage becomes so marked that the lymphatics can no longer handle the load and pulmonary edema ensues. This compound should in the future be of great value to physiologists in elucidating the exact mechanism of pulmonary edema.

In the third lecture Drinker discusses the effect of breathing movements upon lymphatic flow in pulmonary tissue. Cleverly designed experiments are described which indicate that increased inspiratory and expiratory efforts augment the flow of lymph from the lung. The importance of position as the cause of blood stasis and atelectasis is emphasized. The author warns against the dangers of allowing stasis to develop in hospitalized patients and mentions some of the more practical methods of avoiding this usually unnecessary complication.

The fourth and final lecture deals with a discussion of "Preventive and Therapeutic Measures in Asphyxiating Pulmonary Disease." This lecture opens with two delightful paragraphs, the first of which deals with the relationship between liberalism and the scientific mind and the second which contains the following description of the characteristic Harvard Professor: "He wears a red necktie; his trousers do not fit; and he is invariably right!" There follows a scholarly but thoroughly practical discussion of the use of oxygen and carbon dioxide in medical therapy. A warning is sounded against the indiscriminate use of morphine and brief comments are made concerning the use of helium and positive pressure oxygen. The physician is urged to employ oxygen early and to educate his patients and their families toward the point of view "that the words oxygen and undertaker are not synonymous."

The final chapter on artificial respiration is a masterpiece and should be read by all medical students and house officers. It not only discusses in some detail the various practical methods of administering artificial respiration, but it also sets forth, in the clearest terms possible, the physiological principles involved in emergency resuscitation. The author points out that mechanical pneumotors are in the main dangerous and ineffective and he emphasizes that immediate manual artificial respiration or mouth to mouth breathing should be employed in respiratory emergencies.

This volume constitutes an important addition to medical literature and maintains the high standards set by previous Harvard University Monographs in Medicine and Public Health.

W. B. WOOD, JR.

The Hair and Scalp. A Clinical Study (With a Chapter on Hirsuties). By Agnes Savill, M.A., M.D. (Glasg.) F.R.C.P.I. Third edition. Williams & Wilkins Company, Baltimore, Md., 1945. Price \$4.75. Cloth with 304 pages and 54 figures.

Agnes Savill has revised and enlarged the new edition of her excellent monograph on the hair and scalp. The opening chapter on the structure and physiology of the hair is a complete presentation of our basic knowledge of this subject. Two excellent chapters follow on canities and the care of the hair. One can challenge, however, the implication that dandruff is infectious.

Permanent waving, singeing, bleaching, and dyeing of the hair are described from the scientific standpoint.

There is an excellent and informative chapter by W. T. Astbury on the molecular structure and elastic properties of hair. It should be read and studied by all physicians who are interested in the hair.

Dr. Savill's descriptions of the many diseases of the hair and scalp are interesting and informative. The relationship of internal diseases and scalp and hair affections are emphasized.

New additions to the present volume are sections on cleansing agents, pigmentation, recent vitamin and endocrine discoveries, congenital defects, rare tumors of the scalp, etc.

Hamilton Montgomery (Mayo Clinic) has evidently inspired additions and changes in the present edition. More credit has been given to American investigators than in previous editions. This will no doubt be further improved in subsequent editions as the volume still lacks reference to some important American contributions.

This book can be highly recommended to all dermatologists and physicians who are at all interested in diseases of the hair and scalp.

RICHARD S. WEISS.

Virus as Organism (Harvard University Monograph in Medicine and Public Health No. 8). By *Frank M. Burnet*, M.D., F.R.S., Director, Walter and Eliza Hall Institute of Research in Pathology and Medicine, Melbourne, Australia. Harvard University Press, Cambridge, Mass. Price \$2.00. Cloth with 134 pages.

In this volume is set down the substance of the Edward K. Dunham Lectures delivered by the author at Harvard University in 1944. In that series of lectures, Dr. Burnet set himself the task of considering the viruses not as macro-molecules or autocatalytic enzymes, or parasites, but as biological entities occupying a place in the cosmos of living things. He explains the basis for his faith that viruses are microorganisms which have evolved by parasitic degeneration from larger microorganisms, many of them in all probability from bacteria. It is his thesis that they still show evidence in their chemical structure of conformity with the general pattern of living material and that their behavior must be interpreted mainly in terms of biological concepts. He examines the general problem of evolution and change in virus disease and the reaction of the host to virus infection.

Spontaneous generation of viruses is dismissed as a concept without supporting evidence and without necessity for a consistent philosophy. The mutation of viruses is discussed and the biological requirements for survival of a mutant strain are listed. It is pointed out that excessive virulence may be just as much of a handicap in the struggle for survival of a virus as inadequate virulence. A perfect parasite is one which does not harm its host too much, multiplies at a rate sufficient to insure its survival, and is able to pass from host to host without excessive difficulty. The special problems of a virus in this respect are pointed out. The author shows that from a biological point of view "virus" must be considered the total mass of virus in existence, not the small collection of particles in a single cell or even a single animal. Herpes simplex, poliomyelitis, psittacosis, the smallpox group, yellow fever, and influenza serve as convenient and important examples of virus disease to which biological concepts are applied.

Dr. Burnet is not only an acknowledged leader in virus research, but also a serious thinker about the larger aspects of disease and a forceful and entertaining writer. This volume will have little interest for the person interested only in the chemistry or physics of viruses, but it is highly recommended to the thoughtful physician as well as the biologist interested in the interrelation of organisms.

ROBERT F. PARKER.

Organic Preparations. By *Conrad Weygand*, Professor at the University of Leipzig. Interscience Publishers, Inc., New York. Price \$6.00. Cloth with 534 pages.

PROTEIN REQUIREMENTS OF ADULTS

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INTRODUCTION

RECENT surveys on the nutritive state of population groups have clearly demonstrated that the intake of many nutrients at levels considerably below National Research Council recommendations are compatible with apparent good health.¹⁻⁴ Estimates of absolute requirements such as have been arrived at for thiamine⁵ are needed if correlation with clinical findings and nutrient intake is to be found, or if dietary intakes are to be more helpful in assessing nutritive state. Also, levels approaching requirement should form the first goal to be sought in rehabilitation work, when food supplies are limited, rather than optimum levels which, under such conditions, may be wasteful.

The recommended allowance of the National Research Council for protein, 1 Gm. per kilogram of body weight per day, is easily obtainable on diets containing foods of animal origin. It is generally conceded that there is a safety factor of about 50 per cent in this allowance, presumably to allow for differences in nutritional value of various proteins. However, a considerable portion of the population (see preceding reviews) consumes less protein than the allowance mentioned, usually of the so-called poor quality proteins, but without ill effects as revealed by clinical examination. Minimum levels of protein, related to the nutritional value of the diet, are needed if dietary surveys are to be used in assessing the relative adequacy of the proteins in ordinary diets.

In view of the fact that there is a paucity of data upon the protein requirements of adults on mixed diets, we have studied nitrogen balance on twenty-six adults who were fed diets low in protein and devoid of animal protein and have investigated the effect of replacing part of the protein in the diet by meat, soy flour, wheat germ, and white bread.

REVIEW OF LITERATURE

Several reviews are available covering protein requirements for adults and the individual papers need not be reviewed. It should be mentioned, however, that in evaluating the nutritional value of the proteins in a diet by nitrogen balance studies, the level of nitrogen fed should be sufficiently low to produce negative balance and should be fed sufficiently long to assure a reasonably constant nitrogen output. Since there is a lag in the change of nitrogen excretion with decreases in dietary nitrogen, short periods will show negative

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balances which are too large. Thus, if either of these conditions is not met, the determined requirement for nitrogen will be too high.

Leitch and Duckworth⁶ estimated the average human requirement to be approximately 50 Gm. of protein per day. This estimate was reached by plotting selected nitrogen balance figures from the literature against protein intake. A regression line was drawn through the positive balances and another through the negative balance figures. The point at which these lines were equidistant from the line of balance was taken as the estimated requirement. The principal criterion of suitability of the data used was that the subjects received the same diet for at least three days preceding the collection of urine and stools. The data were from many sources and no consideration was made of the size of the subjects or the quality of the protein fed. Many of the data show negative balances at such high levels of nitrogen intake that they must be discarded on a logical basis as either inaccurate or unrepresentative of normal adults or usual diets. Further, three days is often inadequate for obtaining a stabilized nitrogen output (see Selection of data). Finally, relatively few data are available at critical levels of intake and practically none at really low levels. Thus the spread of the data is so limited at the levels of real interest as to limit seriously the use of the method employed. The estimate of 50 Gm. of protein per day is undoubtedly high.

The data of Sherman and associates⁷ and of Hindhede⁸ are extensive and approach the criteria of an adequate study, although many of the data were collected on the same subjects and the level of nitrogen intake was usually so adjusted as just to produce negative balance. Thus the spread of the data is limited. By rigorous selection of data, which appears justified, Sherman estimated the protein requirement to be about 40 Gm. per day for a man weighing 70 kilograms. Many of the data, however, were collected on diets consisting almost wholly of a single food—potatoes or wheat or corn. Thus this estimate may be high due to the supplementary action of the proteins of mixed diets.

With regard to supplementary action, Sherman's data clearly show improvement in the nutritive value of cereal diets by the addition of small amounts of milk. However, the differences were apparently not sufficiently large to allow separate estimates of the requirement on the two regimens (cereal alone and cereal plus milk), and data on both types of diets were used in arriving at the estimated requirement of 40 Gm. Considering the levels of nitrogen fed, little justification for recommending high quality proteins can be found in these papers.

Estimates of protein requirements for adults have been arrived at from consideration of the endogenous nitrogen in the urine on protein-free diets. Brody⁹ has made an extensive compilation of the data available and concludes, in agreement with Terroine and Sorg-Matter^{10, 11} and Smuts,¹² that there is a parallelism between basal energy metabolism and endogenous nitrogen excretion. The minimum urinary nitrogen excretion for species ranging from mice to dairy cows appears to be approximately 2 mg. nitrogen (12.5 mg. conventional protein) per calorie of basal heat expended. As pointed out by

Smuts¹² and Terroine¹³ this minimum protein requirement should be replaceable, gram for gram, by a protein with a biologic value of 100 which is completely digestible. On this assumption, the average adult requirement for this high quality protein would be approximately 19 Gm. If one assumes a biologic value as low as 50, the requirement would be satisfied by approximately 38 Gm. Incomplete digestion would similarly raise the requirement. However, few proteins which have been studied have either biologic values or digestibilities as low as 50.¹⁴ On a theoretical basis, then, the requirement is probably between 20 and 40 Gm. In fact, Terroine¹³ concludes that the consumption of a diet of natural foods, rice alone, for example, will adequately cover the protein needs when the caloric needs are satisfied.

The endogenous nitrogen excretion of rats was also found proportional to basal caloric expenditure by Ashworth and Brody.¹⁵ The average figure given by these authors is 1 mg. of nitrogen per 0.71 calories or approximately 1.4 mg. of nitrogen per basal calorie. This figure may be more nearly correct than that of Smuts since it was arrived at using long depletion periods. Protein requirements based on this estimate would thus be approximately 30 per cent less than those given. In this paper, however, we have used Smuts's estimate principally for conservative reasons and because it is based on several species.

INVESTIGATIONS

Subjects.—The subjects used in these studies were twenty-six apparently healthy adults ranging in age from 19 to 50 years. They included five medical students, five home economic students, three graduate nurses, nine students from the Harvard School of Public Health, and four research assistants. Three participated in two different studies. They were all volunteers but were paid while on the study. All of the subjects were selected by personal interview with special consideration for trustworthiness since they continued their regular activities and could not be supervised other than at mealtimes. Each subject collected all urine and stools during the investigation and these were delivered at mealtimes to the investigators in charge. Aliquots of the urine were taken each day and combined. Kjeldahl analyses were made every two, three, or four days, depending upon the investigation. All stools were homogenized with water in a Waring Blender and aliquots handled in the same manner as the urine.

Diets.—All of the diets used in these studies, with the exception of Diet II, were based upon the basic diet, Diet IB, shown in Table I. This diet is devoid of animal protein and is referred to as the all-vegetable diet. The constituents of this diet were selected to approximate in some respects certain low-cost diets. Thus 50 per cent of the nitrogen was supplied by white bread (containing no skim milk solids) and a total of 62 per cent was supplied by cereal products. Potatoes supplied about 13 per cent of the nitrogen, and the total from vegetable products was approximately 30 per cent. The remaining 8 per cent came from fruits. Individual servings of each food were weighed daily and remained constant on each diet. However, considerable ingenuity

TABLE I. PROTEIN CONTENT OF DIETS

FOOD	BASIC ALL-VEGETABLE DIET IB				DIET IIB				DIET II			
	AMOUNT (GM.)	PROTEIN			AMOUNT (GM.)	PROTEIN			AMOUNT (GM.)	PROTEIN		
		(GM.)	PER CENT			GM.	PER CENT			GM.	PER CENT	
Bread (no milk solids)	144	12.000	50.0	50.0	96	8.000	33.3	33.3	-	-	-	-
Rice	24	1.824	7.6	12.0	16	1.216	5.1	8.0	-	-	-	-
Yellow corn meal	12	1.056	4.4		8	0.704	2.9		15	1.320	17.1	17.1
Potatoes	160	3.200	13.3	13.3	107	2.140	8.9	8.9	-	-	-	-
Onions	40	0.560	2.3	16.3	27	0.378	1.6	11.0	50	0.700	9.1	60.5
Carrots (canned)	160	1.600	6.7		107	1.070	4.5		200	2.000	25.9	
Tomatoes (canned)	80	0.800	3.3		53	0.530	2.2		80	0.800	10.3	
Lettuce	80	0.960	4.0		53	0.636	2.7		100	1.200	15.5	
Applesauce (canned)	80	0.160	0.7	8.4	53	0.106	0.4	5.5	300	0.600	7.8	22.1
Orange juice	160	1.440	6.0		107	0.963	4.0		-	-	-	
Peaches (canned)	80	0.400	1.7		53	0.265	1.1		100	0.500	6.5	
Pears (canned)	-	-	-		-	-	-		200	0.400	5.2	
Olives	-	-	-	-	-	-	-	-	20	0.200	2.6	-
Meat	-	-	-	-	34	7.990	33.3	33.3	-	-	-	-
Total (Calculated)		24.000	100.0	100.0		23.998	100.0	100.0		7.720	100.0	100.0

was used in preparing these same foods into dishes which were tasty, interesting, and varied. For example, the corn meal was sometimes served as cereal, sometimes as topping for a vegetable casserole, and sometimes as baked mush with tomato sauce. Individual casseroles were used throughout in preparing dishes which could not be accurately measured after cooking.

The total calorie content of Diet IB was calculated to be 950 calories per day. Additional calories to maintain weight were supplied in the form of rendered butter, cooking oils, cornstarch biscuits, Coca-Cola, ginger ale, jelly, Karo syrup, sugar, and prepared candies. The subjects were allowed these foods freely, but everything eaten was recorded daily. This permitted estimates of the total calorie intakes. The figures for nitrogen intake were also adjusted to include the small amount of nitrogen in these calorie supplements as determined by analysis.

Each day two extra meals were prepared. These meals were placed in waxed cardboard containers in the refrigerator and were homogenized with water the next day in the Waring Blender. Aliquots were saved for Kjeldahl analysis. Figures on nitrogen intake were arrived at from the nitrogen content of the meals to which was added the nitrogen in the calorie supplements. The amount of nitrogen in these supplements was small and therefore each subject, regardless of weight, had essentially the same nitrogen intake. Some coffee, prepared from concentrates to avoid daily analyses, was allowed. This nitrogen was considered to have no nutritive value. It was not included in the calculations of dietary nitrogen and an equivalent amount was subtracted from the urinary nitrogen figures.

Diet IIB (Table I) represents Diet IB supplemented with meat. The amount of each food in Diet IB was decreased by one-third and sufficient meat was added to supply approximately the nitrogen thus removed. Thus, the nitrogen

intake was approximately the same on Diets IIB and IB but about one-third was supplied by meat in Diet IIB. Daily dietary analyses were made as before. The substitution of meat for one-third of the basic diet resulted in a considerable decrease in the caloric content of the diet. This loss was covered by additional amounts of the caloric supplements.

TABLE II. SOURCE AND AMOUNT OF PROTEIN SUPPLIED IN VARIOUS DIETS COMPARED WITH BASIC ALL-VEGETABLE DIET IB

DIET	IB (PER CENT)	MEAT* (PER CENT)	SOY FLOUR† (PER CENT)	WHEAT GERM‡ (PER CENT)	BREAD (PER CENT)	TOTAL AMOUNT AS PER CENT OF IB
IB	100.0					100
IA	125.0					125
IC	150.0					150
IIB	66.6	33.3				100
IID	66.6		33.3			100
IIE	66.6			33.3		100
IIC	66.6				33.3	100
G	56.0	28.3				85
F	100.0	220.0				320

*Ground top of the round prepared in various ways. Thanks are due to Mr. J. H. McManus, Regional Chairman of the American Meat Institute for making arrangements for the purchase of meat.

†Soy, used in biscuits, provided by the A. E. Staley Manufacturing Company, Decatur Ill.

‡Used in biscuits, provided by the Battle Creek Foods, Battle Creek, Mich.

Other diets used are shown in Table II. As can be seen, Diets IA and IC were of the same percentage composition as Diet IB, but each constituent (Table I) was increased 25 per cent and 50 per cent, respectively. Diets IIC, IID, and IIE were the same as Diet IIB (Table I) with bread, soy flour, and wheat germ, respectively, replacing the meat as a source of supplementary protein. Diet G was similar to Diet IIB containing meat, but each constituent including the meat was decreased approximately 15 per cent. Diet F was a high protein diet, IB plus 300 Gm. of meat. Diet H, shown in Table I, was fed at the beginning of Experiment 5 as a preliminary depletion diet. It supplies approximately 8 Gm. of protein per day. Diets J and K consisted of Diet IB plus varying amounts of glycine and cystine. These diets were used in part of Experiment 5 but will not be discussed here as they were concerned with another aspect of this study which will be reported later. Care was taken in weighing the foods, and the nitrogen content seldom varied as much as 0.2 Gm. from the mean value for the experiment.

The vitamin and mineral content of the diets was maintained by supplements* and, with the exception of riboflavin and calcium, was above the National Research Council's recommended daily allowances. Studies on riboflavin and calcium metabolism were made on these diets and will be reported later. The calcium intake varied from 400 to 1,000 mg. per day; the riboflavin intake, from 1.5 to 3.3 mg. per day.

*The supplements included thiamine, riboflavin, nicotinic acid, calcium, phosphorus, iron, and copper. Thanks are due to Abbott Laboratories, North Chicago, Ill., to Merck & Company, Inc., Rahway, N. J., and to United Chemical and Organic Products, Chicago, Ill., for these materials.

Plan of Investigations.—The general plan of the investigations is shown in diagrammatic form in Fig. 1. In most of the studies, the collection of stools and urine was preceded by a three-day adjustment period on the diet and a one-day period between the different diets. In all studies except Experiment 4, three collections of two days or more were obtained on each diet.

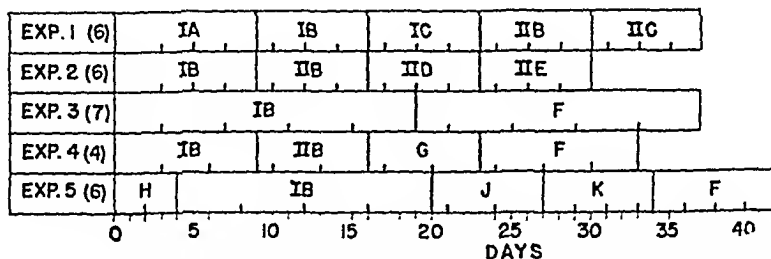


Fig. 1.—General plan of experimental procedure. Figures in parentheses represent number of subjects on each experiment. Small bars show the days included in each analysis of urine and stools.

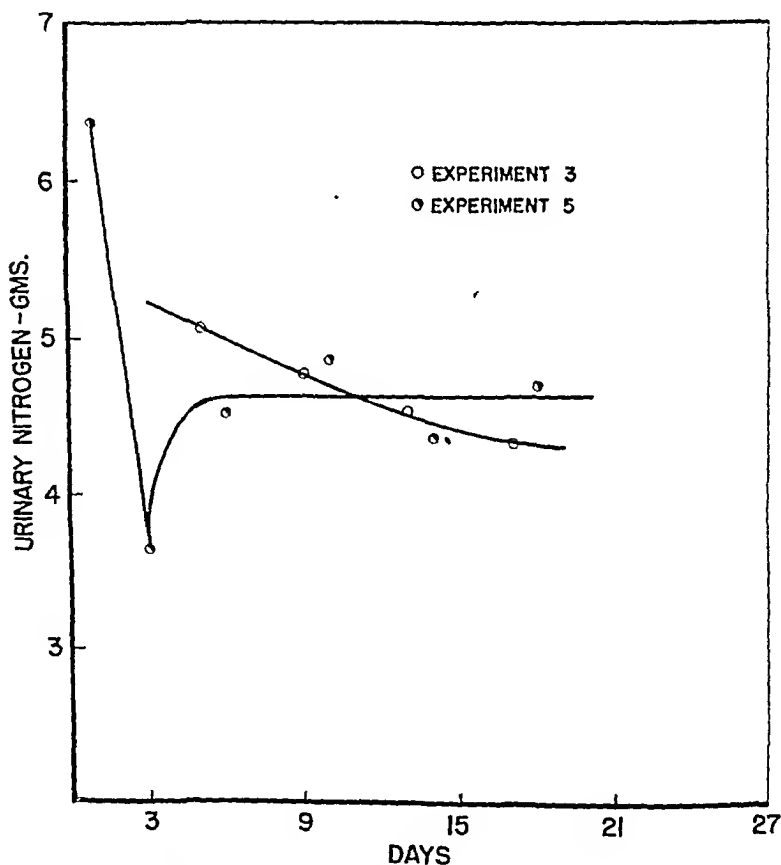


Fig. 2.—Average urinary nitrogen excretion in Experiments 3 and 5.

Selection of Data.—It was apparent from the data, especially in Experiment 3, that there was a great difference in subjects in the time required for stabilization on the diet. Of the seven subjects in Experiment 3, four showed a more or less regular fall in urinary nitrogen per day in the four four-day collection periods amounting to a difference of more than 1 Gm. per day at the start and at the end. The output of one subject was essentially constant and differed only 0.2 Gm. per day at the start and the end. The remaining two fell slightly, showing less than 0.5 Gm. per day difference in the first four days compared with the last four. The average urinary nitrogen excretion of the seven subjects is shown in Fig. 2. Also shown in this figure are the results of urinary excretion in Experiment 5 in which the subjects were fed the low nitrogen diet, Diet H, for four days before receiving the basic diet, Diet IB. Although the results are less consistent than in Experiment 3, it is apparent that there is no tendency for the urinary nitrogen to fall after this depletion period.

In the light of these results, it is certain that three days is far from sufficient for many subjects to reach a stabilized nitrogen output unless a preliminary very low nitrogen period is used. Thus it is recognized that much of the data in the literature probably show urinary nitrogen figures too high for the nitrogen fed and, similarly, that some of our subjects were undoubtedly not stabilized sufficiently. We have attempted to correct this somewhat by using the urinary nitrogen figures during the last four days of those subjects on Diet IB, if these periods were considerably lower than the preceding ones. However, the average fecal nitrogen of the total collection period was always used. Since the supplemented diets always followed the basal diet, we have not selected the data in these periods. It is recognized, therefore, that our estimate of protein requirement may be somewhat *high* and that the order of the experiments places the basic all-vegetable diet, Diet IB, in a less favorable position compared with the supplemented diets.

RESULTS

The results of all of the nitrogen balance studies on the all-vegetable Diet IB are plotted against body weight, surface area, nitrogen intake per kilogram of body weight, and nitrogen intake per square meter of body surface* in Figs. 3 to 6. Both regression lines, one for the best estimate of X (values on the abscissa) and the other for the best estimate of Y (values on the ordinates), are shown. Both regression lines in Fig. 6 are used later in the paper, although in most instances Equation 1 (Fig. 6) is used since we are interested in estimating the value on the abscissa (body size or nitrogen intake) at zero balance.†

It will be recalled that the all-vegetable diet supplied approximately 4.5 Gm. of nitrogen per day (28 Gm. of protein) regardless of body size. This

*Taken from the DuBois body surface chart for height and weight as modified by Boothby and Sandiford (Duncan, G. G.: *Diseases of Metabolism*, Philadelphia, 1942, W. B. Saunders Co.).

†The standard error of the estimate of x ($\sigma_s x$) is given in Figs. 3 to 6. The two small bars on the line of zero balance about regression line 1 show the relative magnitude.

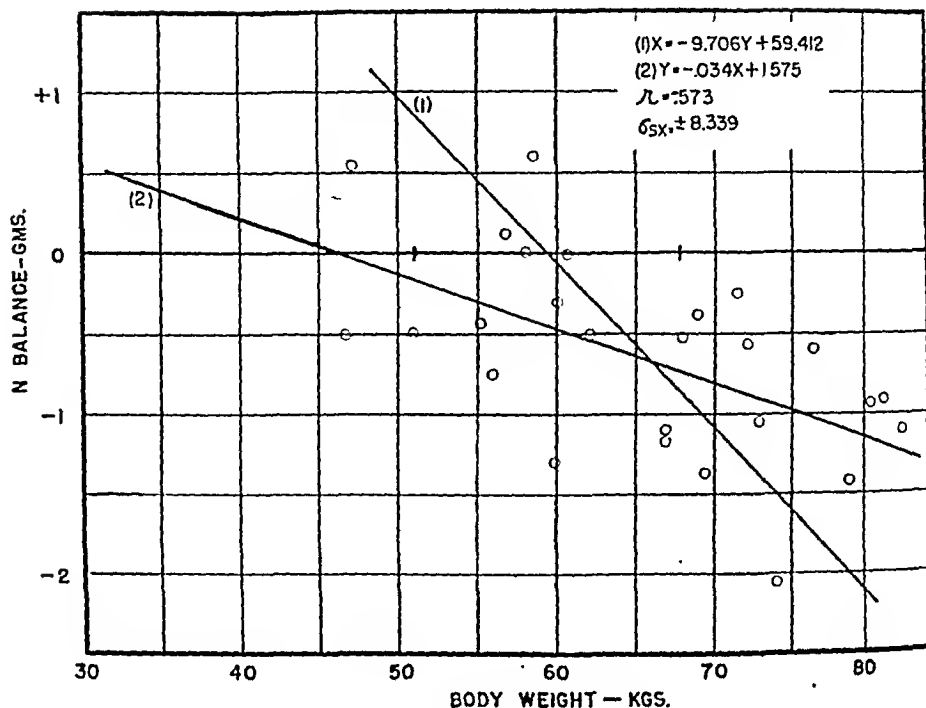


Fig. 3.—Nitrogen balance versus body weight—Diet IB.

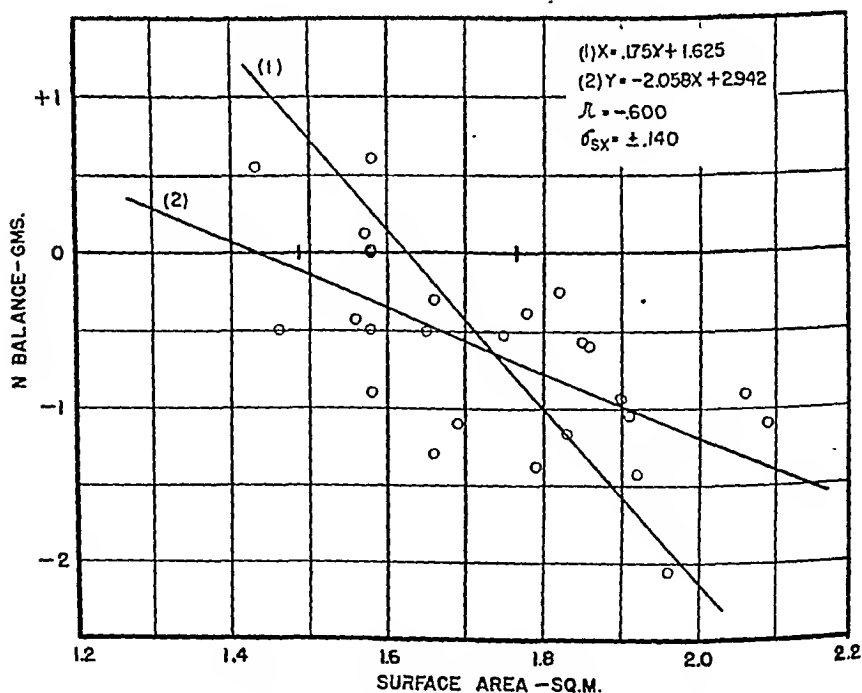


Fig. 4.—Nitrogen balance versus surface area—Diet IB.

value varied slightly with individuals and in different experiments. Thus, one expects a better correlation if intake is calculated per unit of body size. Actually, the correlation is not much improved if calculated per unit of body weight but is considerably better if intake per unit of body surface is used.

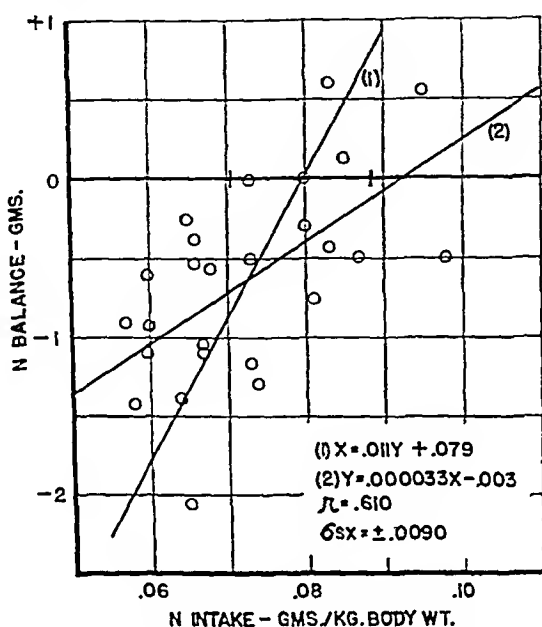


Fig. 5.—Nitrogen balance versus nitrogen intake per kilogram of body weight—Diet IB.

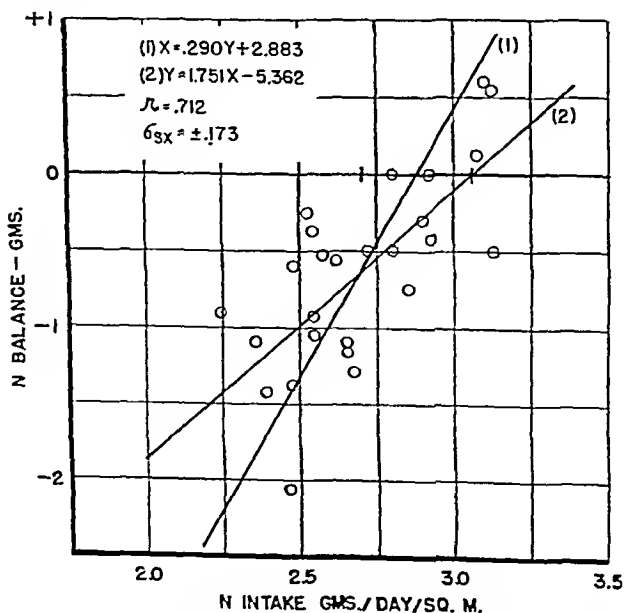


Fig. 6.—Nitrogen balance versus nitrogen intake per square meter of body surface—Diet IB.

The correlation coefficient in the latter instance (Fig. 6) is 0.71 compared with 0.61 on a body weight basis (Fig. 5). Intake per square meter of body surface is thus considered the most appropriate unit in considering protein requirements and is used throughout this paper. This is in agreement with the previous findings of Terroine and Sorg-Matter,¹⁰ Ashworth and Brody,¹⁵ and Smnts.¹² It is realized that the curves cannot be extended unduly. Increases in positive balance will not, for example, continue indefinitely.

According to Equation 1 in Fig. 6, the estimated intake at zero balance is 2.88 ± 0.17 Gm. of nitrogen per square meter of body surface. A man whose weight is 70 kilograms and height, 160 centimeters (surface area 1.73 square meters) would thus require between 4.68 and 5.27 Gm. of nitrogen per day from the all-vegetable diet, Diet IB, which corresponds to from 29.3 to 32.9 Gm. of conventional protein per day. A man of similar weight but 183 centimeters

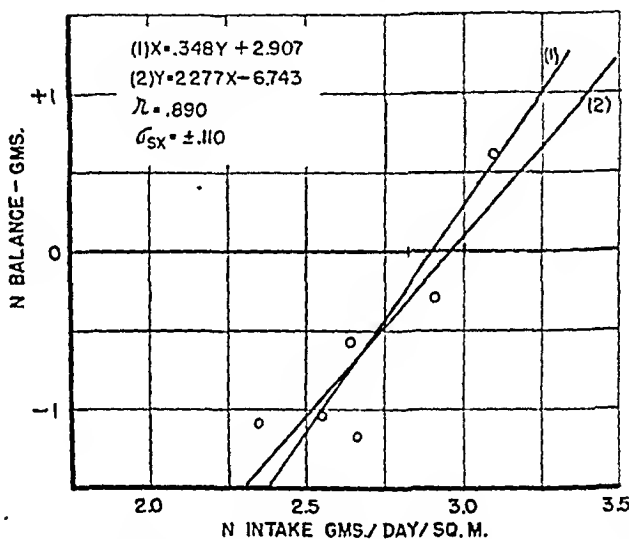


Fig. 7.—Nitrogen balance versus nitrogen intake per square meter of body surface of subjects in Experiment 5—Diet IB.

in height (1.91 square meters) would require from 34.5 to 38.6 Gm. of protein per day. It is of interest to compare the figures obtained on all the subjects with those in Experiment 5 where the subjects were apparently depleted by the period on Diet H. These results are plotted in Fig. 7. The correlation is good although the number of subjects is small. The requirement here is found to be 2.91 ± 0.11 Gm. per square meter of body surface per day which is essentially the same figure found. It appears, therefore, that the requirement arrived at in the previous data is not greatly overestimated, although ideally the studies should have been of longer duration.

The data obtained in Diet IIB, in which one-third of the protein was supplied by meat, are plotted against nitrogen intake per square meter of surface area in Fig. 8. This line crosses the line of balance at 2.73 Gm. nitrogen per square meter, which would indicate a protein requirement of 29.5 Gm. for a man

with a body surface of 1.73 square meters. However, the correlation is poor and many of the subjects were in positive balance, some without appreciable depletion during the preceding period on Diet IB. We may thus expect this estimate to be high and better results would have been obtained at a lower nitrogen intake. This was done in Experiment 4 using Diet G. The data on these subjects, who had been on the experimental diet for sixteen days previously, are plotted in Fig. 9. Only one regression line is shown since the two

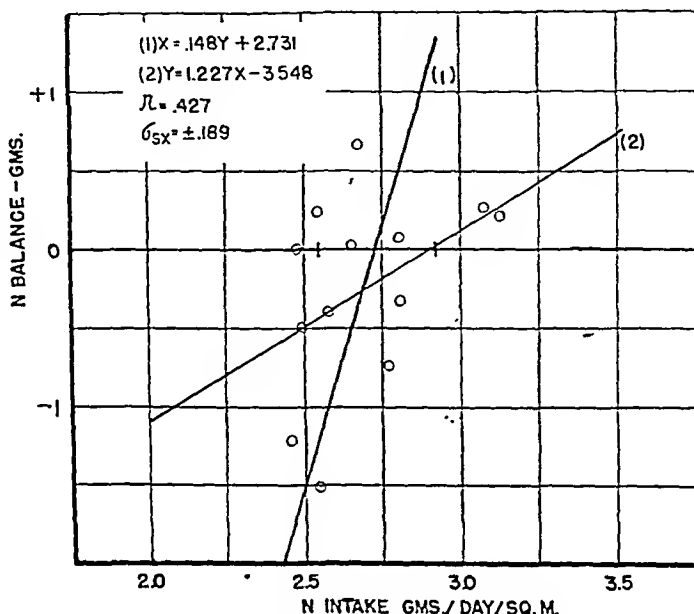


Fig. 8.—Nitrogen balance versus nitrogen intake per square meter of body surface—Diet IIB.

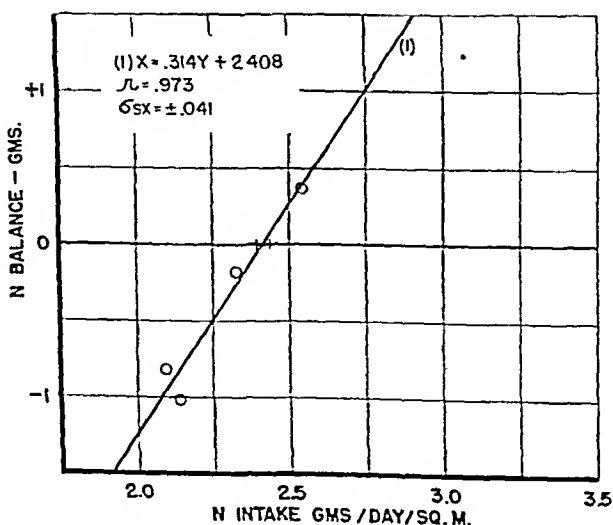


Fig. 9.—Nitrogen balance versus nitrogen intake per square meter—Diet G.

are practically identical. The estimated requirement is 2.41 ± 0.04 Gm. nitrogen per square meter. Although based on only four subjects, this estimate would give a protein requirement of from 25.7 to 26.5 Gm. per day for a man with a body surface of 1.73 square meters. We believe this estimate to be more nearly correct for the diet containing meat.

Insufficient data are available to calculate the comparable requirement on Diets IIC, IID, and IIE. We show rather a summary of the data in Table III where the balances obtained are compared with the balance which would result from the same intakes on Diet IB as calculated by equation 2 derived from the data in Fig. 6:

$$\text{Balance} = 1.751 \text{ Intake per square meter} - 5.362$$

The variations in balance found in the different diets and those obtained on Diet IB were compared by Fisher's "t" test. It is seen that the improvement on Diet IIB is highly significant. There can thus be no doubt that the diet with meat is an improvement over the all-vegetable diet. It appears like

TABLE III. NITROGEN BALANCE ON SUPPLEMENTED DIETS COMPARED WITH BASIC ALL-VEGETABLE DIET

DIET	SUPPLEMENT	WEIGHT (KG.)	AREA (SQUARE METER)	URINARY NITROGEN (GM.)	FECAL NITROGEN (GM.)	AVERAGE NITROGEN INTAKE (GM. PER SQUARE METER)	N BALANCE (EXPERIMENTALLY DETERMINED) (GM.)	CALCULATED N BALANCE ON DIET IB AT SAME INTAKE† (GM.)	DIFFERENCE (GM.)
IB	None	65.5	1.73	4.29	1.04	4.70	2.72	-.63	-.03
IIB	Meat	63.5	1.69	3.87	0.90	4.53	2.68	-.24	+.45
IIC	Bread	60.2	1.64	4.33	0.83	4.65	2.83	-.51	-.10
IID	Soy flour	60.7	1.67	4.07	1.09	4.27	2.56	-.89	-.01
IIE	Wheat germ	60.7	1.67	3.43	1.02	4.34	2.60	-.11	+.70

*Probability of difference from Diet IB being due to chance.

†From Equation 2 in Fig. 6.

that Diet IIE (wheat germ) would also show significant improvement if more data were available, although in the data at hand the differences are on the borderline of significance. Soy flour and bread apparently do not improve and depress nitrogen balance in these experiments.

Digestibility.—As shown in Fig. 1, three diets, IA, IB, and IC, which were of identical percentage composition but which varied in amount, were fed to six of the subjects in Experiment 1. Diet IA contained 25 per cent more of each constituent than IB, and IC, 50 per cent more. The differences in fecal nitrogen on these three diets allow an estimate of the digestibility of the nitrogen in the diet. In Fig. 10 is shown the average fecal nitrogen on these three diets. It is fortuitous that the three points fall exactly on a straight line. An extension of the line to zero nitrogen intake may be taken as an estimate of the metabolic nitrogen (fecal nitrogen on a nitrogen-free diet) of these subjects whose average weight was 61 kilograms.

If 0.395 Gm. be taken as the metabolic nitrogen, then the excess nitrogen excreted was $0.950 - 0.395 = 0.555$ Gm. on Diet IB, 0.710 on Diet IA, and

TABLE IV. DIGESTIBILITY OF PROTEIN SUPPLEMENTS AND OF SUPPLEMENTED DIETS

	DIET				
	IIB	IIC	IID	IIE	F
Supplement	Meat	Bread	Soy flour	Wheat germ	Meat
Total N intake (Gm.)	4.53	4.65	4.27	4.34	14.7
Estimated N from Diet IB	3.00	3.00	3.00	3.00	3.00
Estimated N from supplement	1.53	1.65	1.27	1.34	11.7
Average fecal N (Gm.)	0.90	0.83	1.09	1.02	1.55
Fecal N from Diet IB (Fig. 10)	0.77	0.77	0.77	0.77	0.77
Fecal N from supplement	0.13	0.06	0.32	0.25	0.78
Digestibility of supplement (per cent supplement absorbed)	91.5	96.4	74.8	81.3	93.3
Fecal N less metabolic N (Gm.)	0.5	0.43	0.69	0.62	1.15
Absorbed nitrogen (total diet)	4.03	4.22	3.58	3.72	13.55
Digestibility of total diet protein (per cent total N absorbed)	89.0	90.8	83.8	85.8	92.2

0.855 on Diet IC. These represent 12.4, 12.5, and 12.3 per cent, respectively, of the nitrogen ingested. Subtracting this excess excretion from 100 gives a digestibility of approximately 87.5 per cent.

If it be assumed that the metabolic nitrogen of all the subjects is approximately the same as for the subjects who supplied the data in Fig. 10, it is possible to estimate the digestibility of the various diets and supplements. These calculations are shown in Table IV. It is estimated that 3.00 Gm. of the total nitrogen in the diet were supplied by Diet IB. This, according to Fig. 10, would produce 0.77 Gm. of fecal nitrogen.* The difference between this figure and the total fecal nitrogen represents the fecal nitrogen or un-

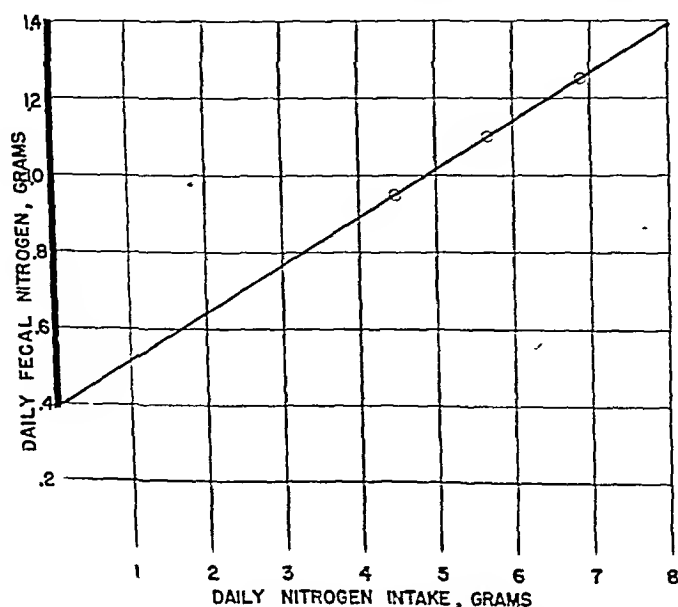


Fig. 10.—Fecal nitrogen versus nitrogen intake on all-vegetable diets—Diets IA, IB, and IC.

*This figure may be somewhat too low, since on Diets IIB, IID, and IIE the caloric supplements were increased.

digested nitrogen from the supplement, and thus the per cent of the supplement digested can be calculated.

Similarly, if the metabolic nitrogen be subtracted from the total fecal nitrogen, the fecal nitrogen from the diet is obtained. Subtracting this from the ingested nitrogen gives the dietary nitrogen absorbed. The figures obtained (Table IV) show no great differences in the digestibility of the various diets. As might be expected, the diets with meat and bread are slightly more digestible than the basic diet, while those containing soy flour and wheat germ are somewhat less. On the other hand, there appear to be marked differences in the digestibility of the supplements. Meat and bread appear highly digestible, while soy flour and wheat germ are considerably less so. Digestibility figures as determined with rats are: beef round, 96; bread, 100 (calculated from data of Mitchell and Carman¹⁶); and soy flour, 85.¹⁷

TABLE V. EFFECT OF PROTEIN SUPPLEMENTS ON BIOLOGIC VALUE OF AN ALL-VEGETABLE DIET

	DIET				
	IB	IB	IIC	IID	IIE
Supplement	None	Meat	Bread	Soy flour	Wheat germ
Absorbed N (Gm.)	4.11	4.03	4.22	3.58	3.72
Urinary N (Gm.)	4.29	3.87	4.35	4.06	3.43
Basal calories	1580	1541	1475	1504	1504
Calculated endogenous N (Gm.)	3.16	3.08	2.95	3.01	3.01
Absorbed N excreted (Gm.)	1.13	0.79	1.27	1.05	0.42
Absorbed N retained (Gm.)	2.98	3.24	2.95	2.53	3.30
Biologic value $\frac{(\text{Retained N})}{(\text{Absorbed N})}$	72.5	80.4	70.0	70.6	88.8

Estimated Biologic Value.—Using the above determined figures for absorbed nitrogen, an estimated biologic value was calculated. For the calculation of biologic value, a figure for endogenous nitrogen (the urinary nitrogen on a nitrogen-free diet) is needed. We have used a figure of 2 mg. of nitrogen per basal calorie per day based on the data of Smuts.¹² The basal calorie requirement used is a weighted average for the subjects, assuming 37 calories per square meter per hour for women and 39.5 calories per square meter per hour for men. The calculations are shown in Table V. These figures are presented primarily for comparison since they are based on an assumed figure, and the subjects were not completely depleted. However, the figures are probably minimum biologic values and again indicate no change in the nutritional value of the diet through the addition of soybeans or more bread, while meat and wheat germ cause an improvement in biologic value. As mentioned previously, the data with wheat germ are too few to indicate the significance of the results with this supplement. The improvement in biologic value of the low protein all-vegetable diet by replacing one-third of the protein with meat is highly significant. Since biologic values are not additive, figures from the literature for separate proteins cannot be used to calculate values for mixed diets, nor can estimates of the biologic value of the individual supplements be made from data on mixed diets.

Relation of Fecal Nitrogen to Body Weight, Caloric Intake, and Expulsion Time.—The data available from these studies provide some information as to the causes of differences in fecal nitrogen on different diets and among individuals. Mitchell^{18, 19} showed with rats that the fecal nitrogen was dependent upon the amount of food consumed. It is apparent from Fig. 10 that this is also true in these studies, and this must of course be true if digestibility for any given diet is constant at different levels of intake. On the other hand, Reifenstein and associates²⁰ conclude that fecal nitrogen is essentially constant over a wide range of protein intakes. These authors point out that the custom in some laboratories to assume that fecal nitrogen represents about 10 per cent of the intake gives figures much too high on high protein diets. These authors believe that the fecal nitrogen is more or less constant, at about 1.28 Gm. per day.

A comparison of the digestibility figures for meat (Table IV) in Diets IIB and F which contained meat in widely different amounts shows reasonably good agreement, 91.5 per cent and 93.3 per cent, respectively. The slightly better digestibility on the higher level of meat may be due to a lower intake of bulk, as the cornstarch biscuits which contained 0.6 per cent algin* were consumed in smaller amounts on this diet. It is not to be expected that fecal nitrogen would parallel nitrogen intake, since metabolic nitrogen constitutes a large and supposedly constant fraction if the digestibility of the diet is high. However, unless digestibility is dependent on nitrogen intake, an increase in fecal nitrogen must occur with increasing intakes. Mitchell²¹ has pointed out that different samples of the same foodstuff may give rise to different values for fecal nitrogen, varying with the amount of indigestible carbohydrate, not only because of the bulk thus provided, but also because such constituents may protect in varying degree against enzyme action in the gut. Similarly, the method of preparation and degree of mastication are probably important. We feel, for example, that high fecal nitrogen figures obtained in at least one of our subjects may be partially related to poor mastication due to malocclusion.

The total fecal nitrogen has been plotted against body weight and against caloric intake (figures not shown), but the correlation in both was poor ($r = +0.28$ for body weight; $r = +0.28$ for caloric intake). On the other hand, we have calculated the correlation of fecal nitrogen to body weight for the data presented by Cahill and associates²² and Murlin and co-workers.²³ Correlation coefficients of $+0.73$ and $+0.77$ were obtained, whereas practically no correlation with caloric intake was found.

One important cause for variations in fecal nitrogen was shown by correlating the time required for carbon markers to pass through the body (expulsion time) with the average amount of fecal nitrogen. In general, the shorter the expulsion time, the higher were the values obtained for fecal nitrogen. One subject had an expulsion time of only seven hours and a high fecal nitrogen of 1.7 Gm. per day, while another on the same diet had a fecal nitrogen of only 0.4 Gm. per day and an expulsion time of sixty-two hours.

*A sodium alginate preparation supplied by the Bushway Ice Cream Division, General Ice Cream Corporation, Somerville, Mass.

These subjects demonstrate extremes encountered and should provide a feeling of caution in the application of average figures for fecal nitrogen. Variations in bowel habits, and the effect of diet on bowel habits, are obviously important factors and probably account for greater variation in our data than in the figures presented by Cahill and associates²² and Murlin and co-workers.²³

Condition of the Subjects.—All of the subjects were well aware of the purpose of the studies. Some of them were interested in, and well informed on, the subject of protein deficiency. After a few meals, they formed a group highly subjective to suggestion. They were undoubtedly influenced by frequent questioning to determine changes in physical and mental condition. Many of them reported unusual fatigue and hunger in the late morning and afternoon, and many reported they needed more sleep than usual. These symptoms of postprandial hypoglycemia are suggestive of the finding of Thorn and associates²⁴ that meals high in carbohydrate and low in protein and fat are likely to be followed in two or three hours by hunger and fatigue. However, there is no doubt that those affected most adversely were those who most expected these results. The more stolid individuals and three subjects who participated in two different studies and were well accustomed to the routine were not at all sure that the low protein diet was detrimental in any way. Although a questioning of the subjects thus indicated some physical deterioration, the significance of this is doubtful.

Physical examinations, usually at weekly intervals, failed to reveal any ill effects of the diets, and body weight was maintained. Blood samples were taken at these times (except in Experiments 1 and 5) and analyses for hemoglobin, hematocrit, total plasma protein, albumin, and globulin were made, the latter three determinations by the Kjeldahl method. The results of these analyses in Experiments 2, 3, and 4 are presented in Fig. 11.

The significance of variations in different periods have been compared using the "t" test. In Experiment 2, the significant changes are a fall in serum albumin and total protein during the second period and a consistent rise in serum globulin during the protein supplemented periods. Similarly in Experiment 4, there was no significant change during the first nine days on the basic diet but a marked fall in albumin and rise in globulin during the next seven days when the subjects were on the low protein diet, in which one-third of the protein was furnished by meat. During the last ten days of the experiment on the high protein, high meat diet, there was an increase in plasma albumin and total plasma protein and a decrease in globulin. In Experiment 3, in which the low protein all-vegetable diet was continued for nineteen days, both the albumin and globulin decreased. The results of the blood analyses in these three experiments are quite consistent. In general, they show a decrease in albumin during the second week of the low protein period regardless of whether the protein source is all vegetable or one-third of it comes from meat. It is of interest to note the rise in globulin on the low protein diet containing meat (Diet IIB) compared with the fall on the basic diet in Experiment

3. Is it possible that the quality of the protein in the diet as well as the quantity has a directing influence upon the type of blood proteins synthesized and that on a diet in which the protein is reduced to near the absolute requirement, the diet containing meat favors globulin synthesis? At a still lower level of nitrogen intake (Diet G, Experiment 4), the globulins did fall while the subjects were on the meat supplemented diet. During the following period on the high meat diet, the globulin continued to fall toward a normal level as the albumin increased. The fall in albumin during the second period in Experiments 2 and 4, when one-third of the protein in the low protein all-vegetable diet was replaced by meat (Diet IIB), is not interpreted as an effect of meat supplementation, but more likely the nine days preceding, on Diet IB, were insufficient to produce the fall. This is strongly suggested by the fall in albumin over a nineteen-day period on Diet IB as shown in Experiment 3. Additional samples during this experiment might have verified this interpretation.

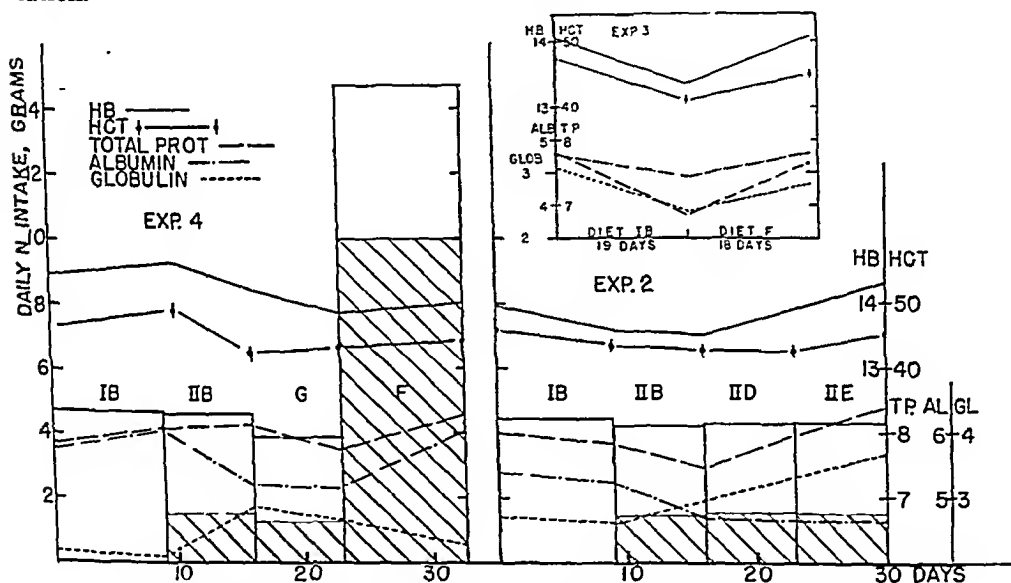


Fig. 11.—Changes in blood constituents throughout Experiments 2, 3, and 4. Areas with diagonals represent levels of nitrogen supplied by supplements.

In all experiments there was a tendency for the hematocrit to fall, which, in general, was reflected by the changes in hemoglobin concentration. Statistically, however, only the decrease during Experiment 3 was significant. All the blood constituents rapidly revert toward normal on the high protein diet.

Previous work on experimental protein deficiency in the rat²⁵ has shown that there is considerable decrease in plasma volume before there is any significant lowering of plasma protein concentration. Hence, plasma volume studies, using the Evans blue dye, were done on four of the subjects in Experiment 2 and on five of the subjects in Experiment 3. Measurements were made at the end of the low protein period and again at the end of the high

protein period. The plasma volume did not vary significantly over these periods, and the average of the nine subjects at the end of the all-vegetable low protein diet was 3,165 ml. as compared with 2,933 ml. at the end of the high protein period.

Basal metabolism and R.Q. measurements were made on all subjects in Experiment 4 at the end of the last low protein period (twenty-three days) and four days after being on the high protein high meat diet. The changes were slight and in line with what might be expected. Average figures of 34 calories per square meter body surface per hour at the end of the low protein period and 37 calories after four days on the high protein diet were obtained in the metabolism tests. The R.Q. decreased from 0.85 to 0.80 by changing to the high protein intake.

DISCUSSION

It has been pointed out previously in this paper that estimates of protein requirements are most likely to err on the side which gives high values, principally because stabilized nitrogen outputs may not be obtained except in very long experiments. Lack of stabilization leads to negative balance figures which are too large and, in calculations on biologic value, to figures for retention which are too small. From the standpoint of practical nutrition, this is a fortunate occurrence, and estimates of protein requirements such as we have determined may be applied with little danger that they are low.

It is of interest to compare the protein requirement as experimentally determined in these studies with those calculated from biologic values and digestibilities. The requirement, using 2 mg. of nitrogen (0.0125 Gm. conventional protein) per basal calorie as the endogenous metabolism, may be written:

$$\text{Protein requirement in Gm.} = \frac{0.0125 \times \text{basal calories} \times 10,000}{\text{Biologic value} \times \text{digestibility}} + \text{Metabolic N} \times 6.25$$

The metabolic nitrogen appears to be small, probably in the range of 0.4 Gm., and equivalent to approximately 2.5 Gm. of protein. Using the figures for basal calories, digestibility, and biologic value from Tables IV and V, the calculated requirements for our subjects compared with those actually determined are given in Table VI. Reasonable agreement is obtained. The fact that the calculated requirements are in most instances somewhat high may indicate, as suggested previously, that our estimates of biologic value or digestibility are low.

It seems appropriate to interject here a brief discussion of biologic values. The significance of biologic values has received extensive and impressive support from the work of Mitchell and his collaborators.^{26, 27} However, the basic supposition of a constant endogenous nitrogen metabolism appears to have been neither proved nor disproved. The findings of Schoenheimer²⁸ necessitate a new interpretation of Folin's original concept but do not appear to negate the possibility of a constant endogenous nitrogen excretion.²⁷ Recently, however, Miller²⁹ has shown, under conditions approximating those used for the determination of endogenous nitrogen excretion, that methionine or cystine

TABLE VI. PROTEIN REQUIREMENT IN GRAMS: COMPARISON OF VALUES CALCULATED FROM BIOLOGIC VALUE AND DIGESTIBILITY WITH VALUES DETERMINED EXPERIMENTALLY

DIET	SUPPLEMENT	BIOLOGIC VALUE	DIGESTIBILITY		CALCU- LATED REQUIRE- MENT (GM.)	EXPERIMENTALLY DETERMINED REQUIREMENT (GM.)
			PER CENT	NET PROTEIN VALUE		
IB	None	72.5	87.5	63.5	23.6	31.2 (Based on Fig. 6)
IIB	Meat	80.4	89.0	71.5	29.4	25.4 (Based on Fig. 9)
IIC	Bread	70.0	90.8	63.5	31.6	31.2 (Based on Fig. 6)
IID	Soy flour	70.6	83.8	59.2	34.3	31.2 (Based on Fig. 6)
IIE	Wheat germ	88.8	85.8	76.2	27.2	25.4 (Based on Fig. 9)

supplements cause a striking fall in the urinary nitrogen output. One may calculate from his figures a biologic value of methionine alone well over 100. The dogs used in the experiment, however, were not at an endogenous level of nitrogen excretion when the methionine was fed. Confirmation of these results using certain proteins has been obtained by Swanson and co-workers²⁰ with rats as the experimental animal. This group of workers has used depletion periods which were more prolonged than those usually used in the determination of biologic values, and their animals are thought to be approximately at an endogenous level of metabolism. Complete evaluation of these results does not appear possible at this time. They do, however, throw doubt on many of the figures for biologic value recorded in the literature; they may indicate that the basic assumption of a constant endogenous nitrogen excretion is untrue. For the present, we prefer to retain biologic value as an exceedingly useful concept in protein studies.

It should be realized that the difference in nutritional value of the high quality and low quality proteins is simply a function of the digestibility and biologic value. It appears to us that modern nutrition instruction places a great deal of emphasis on biologic value but little on digestibility. Most textbooks of nutrition present extensive tables of biologic values without presenting the corresponding digestibility. Both values are equally important in determining the nutritional value of the protein to the individual. Perhaps the best method of comparing the relative nutritional values is to compute the product of the digestibility and biologic value. This figure, called the "net protein value," should represent the per cent of the particular protein actually available to the animal for purposes other than energy. Net protein values for the different diets used in this study are shown in Table VI.

Provided sufficient protein is fed to cover the total body requirement, considering the digestibility and biologic value, any protein source should be satisfactory. These considerations should be valid not only for maintenance in adult nutrition, but also for growth, pregnancy, and wound healing. However, in the latter states there is reason to believe that biologic values may be different and that the relative differences in the nutritional value of various proteins as measured by conventional biologic values and digestibilities may not be the same when there are special demands for protein. Mitchell^{18, 19} finds, for example, lower biologic values as the level of protein in the diet is in-

creased, even though the higher levels were much below optimum for the growing rats used. This indicates a relative inefficient use of proteins for growth. Similar results are obtained in calculating the amount of protein consumed per unit gain of body weight; that is, as the amount of protein is increased, the efficiency decreases.³¹ Studies on amino acid requirements³² also emphasize the inefficiency of amino acid utilization in growth. Whether a similar situation holds for human beings, and in pregnancy and lactation, remains to be studied. Furthermore, it is well known with the rat that it is often impossible to supply sufficient protein for growth from all-vegetable diets. Whereas from 3 to 4 per cent of protein suffices for maintenance, from 15 to 20 per cent is usually required for optimum growth. Foods of high protein content are essential under these conditions.

For adults, however, we may consider some of the lower figures for digestibility and biologic values which have been reported for practical diets. There appears to be evidence that certain diets of the Far East are relatively indigestible. McCay³³ reported digestibilities of 50 per cent, but these diets contained over 700 Gm. of dry rice and a total protein content of 93 Gm. Aron and Hoseon,³⁴ Mason,³⁵ and others have also reported digestibilities between 50 per cent and 90 per cent. More recently Basu and co-workers^{36, 37} have reported digestibility figures of 62, 78, 80, and 89.5 and corresponding biologic values of 75, 66.5, 60, and 59 for several Indian diets. Calculated according to the previous equation, using an average basal caloric expenditure of 1,500 calories, the protein requirement on these four diets would be 42.5, 38.7, 41.6, and 38.0 Gm. per day. It is interesting that the lower digestibility figures given are associated with higher biologic values and vice versa. We have occasionally noted similar results in experimental animals and it is probably related to the effect of differences in intake on the biologic value. For absolute comparison, proteins should be fed at equal levels of digestible protein and also at levels approximating the requirement, since it is here that the biologic values become important.

In calculating requirements from nitrogen balance studies, no consideration is given to the nitrogen loss in sweat, growth of hair, etc. At present, there appears to be insufficient data to evaluate the magnitude of these losses. Furthermore, the value of different proteins in meeting these requirements is unknown. Data obtained on chicks³⁸ suggest that the amino acid requirements for feather growth are considerably different from those for body growth, since birds which feather rapidly have higher arginine and glycine requirements. Poor proteins, as they are generally measured, may meet these requirements. Some allowance must undoubtedly be made above equilibrium requirements in adults, but we believe it to be small and at the present time cannot be estimated in terms of different proteins.

Protein deficiency is known to occur in adults, but it seems likely that this develops in individuals consuming natural foods, only coincident with caloric restriction. Caloric restriction over some time produces a fall in basal metabolism, blood pressure, cardiac rate, and respiratory activity. Whether

this results in a lower endogenous nitrogen and smaller protein requirements has been inadequately investigated. However, it appears logical that protein restriction and starvation produce identical effects regarding protein metabolism. In either case, tissue proteins are destroyed; in the first to meet endogenous requirements and in the second to meet caloric requirements.

No data appear available to indicate the relative value of different proteins in starvation, or indeed whether protein per se is of any value under such conditions. Out of thousands of starved individuals, both children and adults, seen in Europe immediately after the recent war, clinical and laboratory evidence of protein deficiency was found only in those who had consumed grossly insufficient amounts of calories over relatively long periods of time (three months or longer). Ninety-five to 100 per cent of the dietary protein of these starved individuals was furnished by cereal grains and potatoes, and those showing signs of protein deficiency improved simply by consuming more of these foods. Marked variations in the clinical condition of these starved patients with regard to the development of edema suggest a practical problem for research. Starvation edema involves far more than a deficiency of protein—body activity, capillary permeability, efficiency of the circulatory system, blood volume, and other physiologic factors, in addition to calories, and possibly other nutrients are also implicated.

SUMMARY

1. Nitrogen balance studies on twenty-six adults in apparent good health were made using a low protein diet, devoid of animal protein, in which approximately 50 per cent of the protein was supplied by white bread, 12 per cent by other cereals, 30 per cent by vegetables, and 8 per cent by fruit. Adequate calories to maintain caloric equilibrium were supplied by sugars, starch, and fats. It was found that the nitrogen requirement is more closely related to surface area (basal caloric expenditure) than to body weight. The requirement for maintaining nitrogen balance was approximately 2.9 Gm. of nitrogen (18 Gm. conventional protein) per square meter of body surface. A man weighing 70 kilograms would thus require between 30 and 40 Gm. of protein, depending upon height.

2. On the same diet with one-third of the protein replaced by meat, the requirement appears to be about 2.4 Gm. nitrogen (15 Gm. conventional protein) per square meter. Protein requirement is thus about 17 per cent less on this diet than on the all-vegetable diet.

3. Data on digestibility and biologic value were determined and the equal importance of these two measurements for evaluating the nutritional value of a protein is emphasized. High quality proteins are more efficient, but less efficient proteins may serve equally well, provided enough can be fed to cover the requirement considering the specific digestibility and biologic value.

4. The biologic value of the low protein all-vegetable diet used in these studies was increased from 72.5 to 80.4 by replacing one-third of the protein with meat. The digestibility of the two diets was essentially the same.

5. There was no measurable objective change in the physical condition of any of the individuals throughout these studies. Some complained of undue postprandial hunger and fatigue on the low protein all-vegetable diets. These complaints were not present on high protein diets.

6. There were no significant changes in hemoglobin, hematocrit, or plasma volume. Total protein, plasma albumin, and plasma globulin tended to decrease on the low protein all-vegetable diet when fed at a level low enough to produce negative nitrogen balance. The replacement of one-third of the protein in this diet with meat resulted in a prompt increase in the globulin fraction.

7. It is most unlikely that protein deficiency will develop in apparently healthy adults on a diet in which cereals and vegetables supply adequate calories.

8. Considering the experimental data presented in this study, the National Research Council's daily recommended allowance of 70 Gm. of protein for an adult weighing 70 kilograms is most generous and could, if necessary, be reduced to 50 Gm. and still provide approximately 30 per cent margin above requirement.

9. It should be emphasized that the experimental data and the conclusions of this paper apply to adults in apparent good health and do not consider protein requirements in growth, pregnancy, lactation, or disease.

ADDENDUM

Since this paper was written, an important contribution on the protein requirements of adults* has appeared. It is interesting that these authors' data upon a mixed diet which corresponds in some respects to our diet containing meat yield values for the protein requirements similar to those obtained in this study. These authors have calculated protein requirements to include nitrogen losses in sweat, skin, and hair. As discussed previously we believe these requirements have been too little studied to be evaluated at this time, especially in terms of specific proteins.

It is also of interest that these authors find the endogenous nitrogen losses, although determined on only two subjects, to be approximately 1.4 mg. per basal calorie. This figure corresponds closely to that found by Ashworth and Brody on rats rather than the figure of 2 mg. given by Smuts.

We are indebted to Simmons College and particularly to Dr. E. Robb, Professor of Home Economics, for the use of kitchen facilities required in these studies, and to the American Meat Institute, for a grant-in-aid which made this work possible.

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PLASMA PROTEIN LEVELS IN NORMAL INDIVIDUALS

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MORE than 1,500 semi-micro-Kjeldahl determinations of total plasma proteins and albumin in as many different individuals were part of a nutrition survey carried out over a period of five and one-half years in North Carolina (1940-1945). It seemed of value, therefore, to present these extensive data in a series of frequency distributions of persons grouped by race, sex, and age.

The technique used and the characteristics of areas and groups of persons surveyed have been described in previous communications.¹⁻³ It is noteworthy that the entire study pertained to individuals in families who were presumably in ordinary health, living in their customary environment and at their usual occupations. Their nutritional status was that prevailing in this region at the time, and they exhibited no signs of protein malnutrition. The mean intake of protein by adults was from 60 to 90 Gm. daily, varying with year and area of survey. That of men was usually above 80 Gm.

The available data on blood protein levels in presumably normal individuals are not extensive. Peters and Van Slyke⁴ tabulated the normal values for sixteen women and sixteen men. Peters and Eisenman⁵ reported values for fifty-two "normal" persons and found 90 per cent of the levels of total proteins between the limits of 6.3 and 7.7 Gm. per cent. Myers and Muntwyler,⁶ in reviewing the literature to 1940, found normal levels to be from 5.6 to 8.4 Gm. for total proteins, from 3.4 to 5.6 Gm. for albumin, and from 1.35 to 3.55 Gm. for globulin, giving A/G ratios of 1.2 to 2.6. Kagan,⁷ reporting on 150 normal persons tested by the falling drop method, found serum protein levels of from 6.1 to 7.6 Gm. (average, 6.7 Gm.) and considered values greater than 7.5 Gm. or less than 6.0 Gm. as abnormal until proved otherwise. Youmans and co-workers⁸ reported protein levels on a survey of 776 individuals in Tennessee and found the mean level to be 6.95 ± 0.02 Gm. per cent. These levels were based only partly on Kjeldahl determinations, since a large number were obtained by a biuret technique. Robinson and associates⁹ reported a survey on 439 normal subjects in Mexico City (altitude, 8,500 feet) having a mean protein level of 7.75 ± 0.03 Gm. per cent. This high mean must be evaluated in the light of the population surveyed and of its location, and also in the light of the fact that determinations were based entirely on the biuret method.

The present study pertains to by far the largest group of subjects examined to date. The semi-micro-Kjeldahl technique was used throughout. The 1,561 determinations were made on as many different individuals. Since oxalated plasma was used, results are not strictly comparable with those of surveys in which serum was employed.

The surveys reported here were carried out under the auspices of North Carolina State Board of Health, the International Health Division of The Rockefeller Foundation, and Duke University School of Medicine.

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METHODS

Blood collections were made during the period of from 9 to 12 A.M.; there was no request that individuals omit breakfast. A tourniquet was applied and 25 c.c. of blood rapidly withdrawn into a syringe and emptied into a bottle containing dried oxalate (0.1 c.c. of 25 per cent potassium oxalate dried in the oven for from fifteen to twenty minutes). The amount of blood actually withdrawn usually approximated 25 c.c. with a small percentage of unavoidable variations.

Determinations of total protein and albumin were made by a semi-micro-Kjeldahl procedure. The ammonia produced by wet-ashing the samples with a selenium copper sulfate sulfuric acid digestion mixture and superoxal was liberated with alkali and distilled into 10 c.c. of 2 per cent boric acid. Using a methyl red methylene blue indicator, the ammonia was titrated with accurately standardized 0.02 M HCl. For total protein, a sample of 0.2 c.c. of plasma measured with a micro-pipette was digested directly with 3 c.c. of digestion mixture; for albumin, 0.5 c.c. of plasma was precipitated with 14.5 c.c. of 22.5 per cent sodium sulfate solution and allowed to settle overnight at 37° C., and 10 c.c. of a clear filtrate were taken for digestion with the same amount of digestion mixture. Both determinations were carried out at least in duplicate, and only titrations checking within 0.20 c.c. 0.02 N acid were accepted.

DISCUSSION

In Table I are presented the mean values for total proteins and for albumin and globulin separately for eight subdivisions of the surveyed population by age, sex, and color. Since differences in mean levels with respect to age and sex were obviously minor, further analysis was confined to the results obtained for white and colored populations specifically. In Figs. 1 and 2 are presented the observed frequency distributions of the persons in these two population groups according to total proteins, albumin, and globulin.

It is apparent that the frequency distributions are essentially "normal" in type; hence Figs. 1 and 2 contain normal frequency curves fitted to the ob-

TABLE I. PLASMA TOTAL PROTEINS, ALBUMIN, AND GLOBULIN MEAN LEVELS FOR RACE, SEX, AND AGE GROUPS

AGE AND SEX GROUPS	NUMBER IN GROUP		GRAMS PER CENT							
			TOTAL PROTEINS		ALBUMIN		GLOBULIN		A/G RATIO	
	WHITE	COLORED	WHITE	COLORED	WHITE	COLORED	WHITE	COLORED	WHITE	COLORED
Under 15 yr.										
Male	224	114	7.10	7.37	4.65	4.49	2.49	2.93	1.87	1.53
Female	257	113	7.21	7.49	4.78	4.56	2.49	2.99	1.92	1.53
Total	481	227	7.16	7.43	4.72	4.52	2.49	2.96	1.90	1.53
15† yr.										
Male	267	82	7.12	7.39	4.59	4.39	2.58	3.05	1.78	1.44
Female	387	117	7.29	7.50	4.61	4.37	2.73	3.18	1.69	1.37
Total	654	199	7.22	7.46	4.60	4.38	2.67	3.13	1.72	1.40
Grand total*	1135†	426†	7.19	7.44	4.65	4.45	2.59	3.04	1.80	1.46

*Difference of 0.05 in means from Table II due to difference of definition of group centers.

†Albumin determinations totaled 1,098 white, 423 colored; globulin, 1,094 white and 423 colored.

served data, which are represented by histograms.¹⁰ These fitted curves describe the distributions quite well, with the differences due less to systematic shifts than to apparently irrational discrepancies in the number of persons observed in occasional subgroups near the middle of the range of protein values.

When a frequency distribution may be legitimately described by a normal curve, one may easily proceed to calculate the probability that the protein level of one or more persons may fall between any two given limits by using the mean and the standard deviation (sigma) of the curve. On the base scale of each figure are entered the position and the numerical value of the mean as well as the location and values of points exceeding or falling below this value by one, two, and three times the standard deviation.

To facilitate comparisons, the means and standard deviations for each group, together with their standard errors, are collected in Table II. In addition there are tabulated values between which one-half the observations drawn from a "normal" population may be expected to lie (on the basis of the fitted curve) and another pair of values which would be expected to include nineteen-twentieths of all observations. Persons with protein levels falling accidentally outside these wider limits would rarely be found. Conversely, when persons with such values are encountered they should be strongly suspected as having some pathologic condition.

The means in Table II indicate that colored persons possessed a significantly higher level of total proteins and globulin than did white persons, while the albumin level was higher among white persons. The actual differences between the levels of the means, however, were not great in view of the scatter of the observed values as indicated by the standard deviations. The A/G ratio was distinctly higher in the white population (1.80) than in the colored (1.46).

Precipitin tests for syphilis were made on each individual in the survey. The incidence of positive results was low—less than 3 per cent in colored and 1 per cent in white persons. Malaria was not endemic in three of the four

TABLE II. DISTRIBUTION CONSTANTS OF PLASMA TOTAL PROTEINS, ALBUMIN, AND GLOBULIN FOR WHITE AND COLORED POPULATIONS, WITH LIMITS BETWEEN WHICH ONE-HALF AND NINETEEN-TWENTIETHS OF THE OBSERVATIONS MAY BE EXPECTED TO FALL

	TOTAL PROTEINS	ALBUMIN	GLOBULIN
Mean			
White	7.13 \pm 0.013	4.60 \pm 0.011	2.54 \pm 0.013
Colored	7.38 \pm 0.023	4.40 \pm 0.016	2.99 \pm 0.021
Difference and standard error	0.25 \pm 0.026	0.20 \pm 0.019	0.45 \pm 0.025
Standard deviation			
White	0.430 \pm 0.009	0.363 \pm 0.008	0.422 \pm 0.009
Colored	0.469 \pm 0.016	0.320 \pm 0.011	0.429 \pm 0.015
Limits of one-half of distribution			
White	6.84 - 7.42	4.35 - 4.84	2.25 - 2.82
Colored	7.07 - 7.70	4.18 - 4.62	2.70 - 3.28
Limits of nineteen-twentieths of distribution			
White	6.29 - 7.98	3.89 - 5.31	1.71 - 3.37
Colored	6.46 - 8.30	3.77 - 5.03	2.15 - 3.83

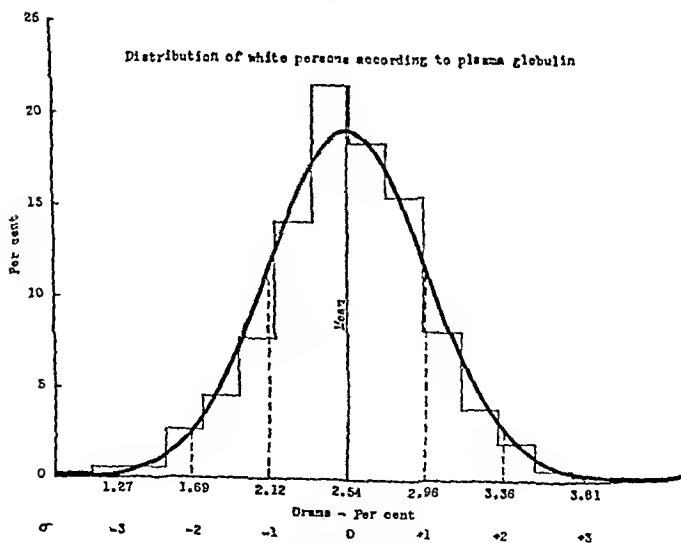
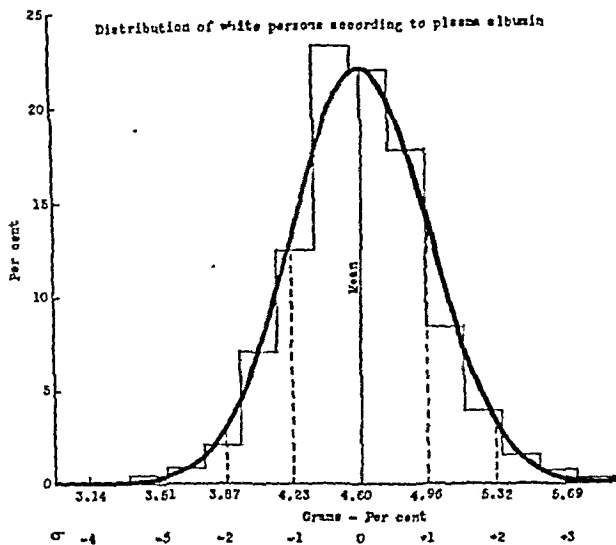
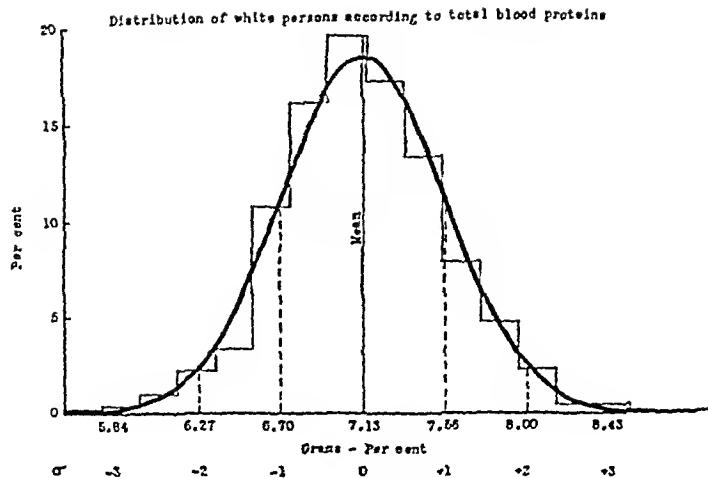


Fig. 1.

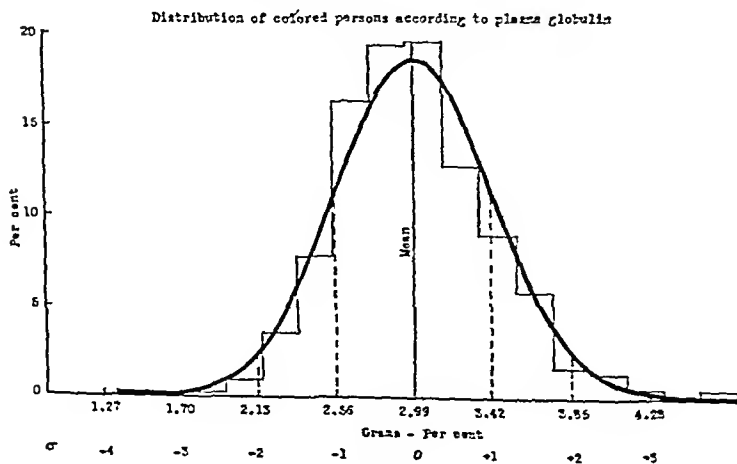
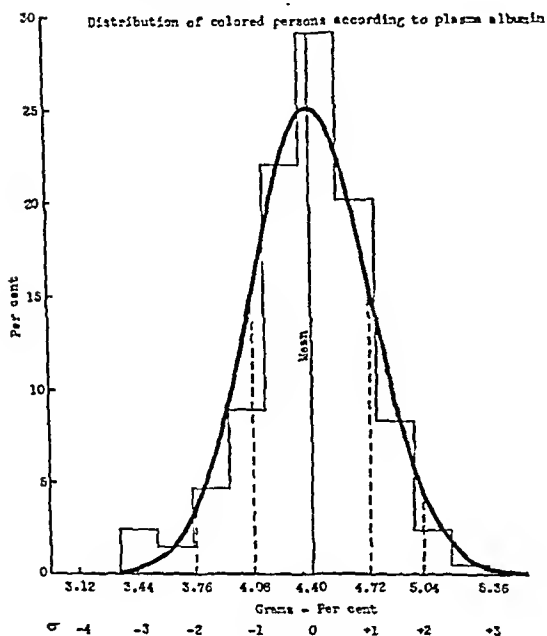
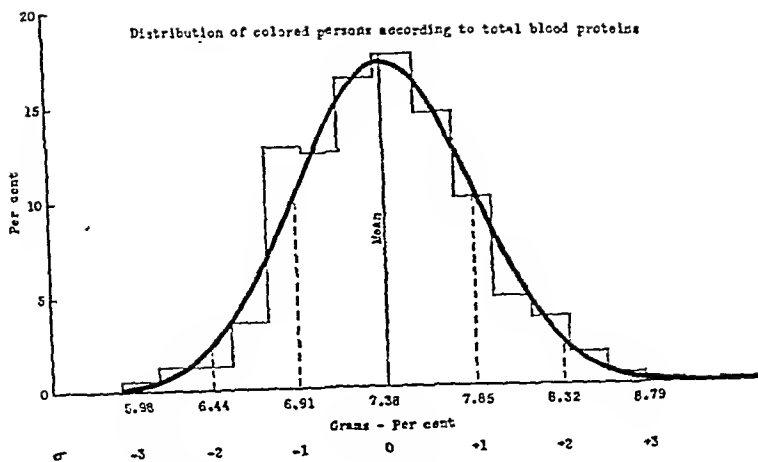


FIG. 2.

counties surveyed. In the other, only one positive (thick) blood smear was found in nearly 900 examinations.

SUMMARY

This paper presents an analysis of blood protein determinations on 1,561 individuals, performed on oxalated plasma by the semi-micro-Kjeldahl method. The individuals, all presumably in normal health, lived on or near the coastal plain in North Carolina.

Blood protein levels of white persons differed somewhat from those of colored individuals, but the factors of age and sex could be disregarded. "Normal" curves well described the distributions of persons in each racial group based on total proteins, or on albumin and globulin separately. The fitted curves are presented in Figs. 1 and 2 and their constants given.

The mean total protein level for white persons was 7.19 Gm. per cent and for colored persons, 7.44. The mean albumin was higher among white than among colored individuals (4.65 and 4.45 Gm. per cent, respectively). The globulin level was, therefore, higher in the colored group (3.04 versus 2.59 Gm. per cent). Mean A/G ratios were 1.80 among white and 1.46 among colored persons. The difference between the races in globulin levels was twice as great as that between age and sex groups of the same race.

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THE TREATMENT OF BACTERIAL ENDOCARDITIS WITH PENICILLIN

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ALTHOUGH the efficacy of penicillin in the treatment of some bacterial infections is well established, its effect in bacterial endocarditis has been more difficult to evaluate. Herewith are presented the results in twenty-eight patients with streptococcal and staphylococcal endocarditis treated with penicillin* in the three-year period from December, 1942, to December, 1945. This report represents a continuation of the cooperative clinical study of the therapeutic effect of penicillin in bacterial infections and includes further observations of three patients previously reported.¹

METHODS

Selection of Patients.†—In this series, all patients with adequate clinical and laboratory evidence of bacterial endocarditis were included, regardless of the patient's condition at the time of admission to the hospital.

Administration of Penicillin.—Although intermittent and continuous methods were used either intravenously or intramuscularly on various occasions, the usual procedure was the intramuscular injection of 40,000 units every two hours day and night. The use of a continuous intramuscular drip was abandoned because of reactions.² Continuous intravenous infusion was employed for the most part only when it was necessary to give massive doses. When bacteremia persisted after several days of therapy, the dose of penicillin was increased.

The duration of treatment was variable, but in the more recent cases it was continued until the patient was afebrile for at least six weeks.

Other Therapy.—Sulfonamide drugs were given to two patients, to one because the infecting organism was resistant to penicillin, and to the other because of a concomitant fungus infection. Anticoagulants were used once and then only in an attempt to maintain a continuous intravenous drip. Supportive measures such as blood transfusions, high calorie diets, and supplementary vitamins were employed freely as indicated.

Observations.—Special efforts were made to maintain complete and accurate records on each patient throughout his stay in the hospital.

With one exception (Case 7), two or more cultures of the blood were positive before the beginning of treatment, and, in that instance, the clinical manifestations were so striking as to justify the diagnosis. In several critically ill

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*The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

†Of the twenty-eight patients comprising this report, twenty-five were treated in the Barnes Hospital and three in the St. Louis City Hospital.

TABLE I. TREATMENT OF STREPTOCOCCUS VITREUS

CASE	AGE	SEX	TYPE OF HEART DISEASE	DURATION OF SYMPTOMS	MURMURS*	NUMBER OF POSITIVE BLOOD CULTURES BEFORE TREATMENT	MAXIMUM COLONY COUNTS PER C.C.	ORGANISM
1	68	Female	Rheumatic	5 mo.	M.I.	6	200	<i>Streptococcus vitreus</i>
2	24	Female	Congenital	1 mo.	P.S., P.I.	7	120	<i>Streptococcus vitreus</i>
3	25	Female	Rheumatic	6 mo.	A.S., A.I., M.S.	5	320	<i>Streptococcus vitreus</i>
4	56	Male	Rheumatic	1 mo.	M.S., A.I., M.I.	4		<i>Streptococcus vitreus</i>
5	38	Male	Rheumatic syphilitic	1½ mo.	A.I., A.S.	2		<i>Streptococcus vitreus</i>
6	22	Male	Rheumatic	2 mo.	M.S., M.I.	6		<i>Streptococcus vitreus</i>
7	50	Male	Rheumatic	1 wk.	M.S., M.I., A.S., A.I.	0†		
8	29	Male	Rheumatic	1 mo.	M.I., M.S.	2		<i>Streptococcus vitreus</i>
9	49	Male	Rheumatic	5 mo.	M.I.	4		<i>Streptococcus vitreus</i>
10	24	Female	Rheumatic	2 mo.	M.I.	4	335	<i>Streptococcus vitreus</i> +
11	56	Female	Rheumatic	6 mo.	M.I., M.S.	6		<i>Streptococcus vitreus</i> +
12	62	Male	Rheumatic	5 mo.	M.I., A.I.	4		<i>Microaerophilic non hemolytic streptococcus</i>
13	20	Male	Rheumatic	6 mo.	M.I., M.S.	4		<i>Streptococcus vitreus</i>
14	55	Male	Rheumatic	6 mo.	M.I., M.S., A.I.	4	120	<i>Streptococcus vitreus</i> ++
15	30	Male	Rheumatic	2 mo.	M.I.	3		<i>Streptococcus vitreus</i>
16	37	Female	Rheumatic	4 mo.	M.S., M.I., A.I.	4		<i>Streptococcus vitreus</i>
17	26	Female	Congenital	4 mo.	P.D.A.‡	4	34	<i>Streptococcus vitreus</i>
18	32	Female	Rheumatic	8 mo.	M.I., M.S.	7	60	<i>Streptococcus vitreus</i>
19	59	Male	Rheumatic	6 mo.	M.I.	4	100	<i>Streptococcus vitreus</i>
20	41	Male	Rheumatic	6 mo.	M.S.	4	35	<i>Streptococcus vitreus</i>
21	22	Female	Rheumatic	10 mo.	M.I., M.S.	4		<i>Streptococcus vitreus</i>
22	15	Female	Congenital	4 days	P.D.A.‡	6	182	<i>Streptococcus vitreus</i>

*M.S., Mitral diastolic murmur; M.I., mitral systolic murmur; A.S., aortic systolic murmur; A.I., aortic diastolic murmur; P.S., pulmonic systolic murmur; P.I., pulmonic diastolic murmur; P.D.A., patent ductus arteriosus and for murmur of patent ductus arteriosus.

†See Appendix.

‡Many negative blood cultures including those obtained by sternal and arterial routes.

§Also *Actinomyces bovis* five times.

patients in whom the clinical findings were indicative of bacterial endocarditis, three or four blood cultures were drawn over a period of several hours, after which therapy with penicillin was instituted. In each of these patients the blood cultures were subsequently found to be positive and the diagnosis confirmed. Frequent blood cultures were obtained throughout and immediately following the course of therapy.

BACTERIAL ENDOCARDITIS WITH PENICILLIN

AMOUNT OF DAILY DOSE (UNITS)	ROUTE	DURATION OF TREATMENT (DAYS)	TOTAL PENICILLIN (UNITS)	RESULT
10,000	I.M.	7	1,700,000	Died on the seventh day of therapy; autopsy revealed rheumatic mitral disease with active vegetations
10,000-700,000	I.M., I.V.	73	13,783,500	Well eighteen months after therapy
10,000-720,000	I.M.	52	17,580,000	Well nineteen months after therapy†
10,000	I.M.	25	5,980,000	Died one month after therapy†
10,000	I.M.	3	960,000	Died on third day of therapy; autopsy showed aortic and mitral rheumatic valvulitis with active vegetations, bicuspid aortic valve, syphilitic aortitis
10,000	I.M.	19	5,850,000	Well fourteen months after therapy†
10,000-1,200,000	I.M.	56	38,600,000	Well ten months after therapy; one episode of cardiac decompensation
10,000-500,000	I.M.	60	23,300,000	Well ten months after therapy
10,000	I.M.	3	580,000	Died on third day of therapy probably from cerebral embolus; autopsy revealed rheumatic mitral disease with active vegetations; brain not examined
10,000-5,000,000	I.M., I.V.	49	167,755,000	Alive and improved; blood cultures positive on discharge†
10,000-960,000	I.M.	11	6,720,000	Died on eleventh day of therapy; autopsy revealed mitral and tricuspid disease with active vegetations on both
10,000-480,000	I.M.	35	12,060,500	Well eight months after therapy
10,000-720,000	I.M.	50	22,462,000	Well eight months after therapy
10,000-1,200,000	I.M., I.V.	28	22,310,000	Died of cardiac insufficiency twenty-eight days after therapy; resistant organism; autopsy revealed aortic and mitral rheumatic disease with active vegetations
10,000-480,000	I.M.	40	15,320,000	Well six months after therapy
10,000-480,000	I.M.	42	14,500,000	Died one month after therapy in acute cardiac failure; no evidence clinically of recurrence; no autopsy
10,000-960,000	I.M.	65	29,580,000	Well four months after therapy
10,000	I.M.	43	20,200,000	Well four months after therapy
10,000-480,000	I.M.	74	32,880,000	Well three months after therapy
10,000-960,000	I.M.	61	32,600,000	Well three months after therapy
10,000	I.V.	4	4,150,000	Died on the fourth day of therapy; no autopsy†
10,000	I.M.	68	32,160,000	Well two months after therapy

†Ligation of patent ductus arteriosus by Dr. Evarts D. Graham during therapy.

†Ligation of patent ductus arteriosus by Dr. Karl Poppe during therapy.

†, Relatively resistant to penicillin (see Appendix).

††, Relatively resistant to penicillin; not inhibited by concentrations less than 2.5 units per cubic centimeter.

The following technique for blood cultures was used:

Twenty-three cubic centimeters of blood were drawn with aseptic precautions and transported from the bedside to the laboratory in a flask containing 3 c.c. of a 4 per cent solution of sodium citrate. A flask containing 100 c.c. of beef infusion broth was inoculated with 10 c.c. of blood, and for growth under reduced oxygen tension 10 c.c. of blood were placed in a flask of thioglycollate broth.³ The latter medium was contained in a Florence flask of 125 c.c. capacity in order to minimize the area of the surface in contact with the air. In our ex-

perience, growth of *Streptococcus viridans* was especially prompt and abundant in thio-glycollate broth. In addition, pour plates were made with 1 and 2 e.c. amounts of blood. Each 100 e.c. of medium contained 5 mg. of para-aminobenzoic acid⁴ and, during the latter part of the study, 1 mg. of cysteine hydrochloride was also added in order to inactivate penicillin carried into the culture from the blood of the patient.^{5, 6} The cultures were observed daily and subcultures made when indicated.

In vitro sensitivity to penicillin⁶ of many of the streptococcal strains was tested† with various methods, but the results of repeated experiments were not consistent enough to state reliably the number of units of penicillin needed for antibacterial action on a given strain. However, it was readily recognized and confirmed that strains from two patients were relatively resistant.

The clinical condition of all surviving patients has been evaluated frequently and information regarding fever, embolic phenomena, anorexia, weight loss, and malaise has been especially sought. Blood cultures have been obtained whenever possible. Except in two instances, post-mortem examinations have been carried out on the patients who have died.‡

RESULTS

Streptococcal Endocarditis.—Twenty-two of the patients had streptococcal endocarditis and significant data concerning them are summarized in Table I. The number of survivors with penicillin therapy and the duration of the period since termination of treatment are shown in Tables II and III. Illustrative case histories are presented in the appendix. It should be noted that a complete course of therapy was given to fifteen patients, and of these, thirteen are now living and well (Appendix, Case 6). On the other hand, of the five patients who died before the completion of therapy, three died within the first four days of treatment (Cases 5, 9, and 21) and the other two within eleven days (Cases 1

TABLE II. RESULTS OF PENICILLIN TREATMENT OF PATIENTS WITH BACTERIAL ENDOCARDITIS

ORGANISM	NUMBER OF CASES	LIVING AND WELL	FAILURES	
			LIVING BUT WITHOUT ARREST OF INFECTION	DIED
Streptococcal	22	13	1	8
Staphylococcal	6	5	0	1
Total	28	18	1	9

TABLE III. LENGTH OF FOLLOW-UP IN PATIENTS NOW CLINICALLY WELL

Eighteen to twenty-four months	4
Twelve to eighteen months	1
Six to twelve months	6
Three to six months	5
One to three months	2
Total	18

*A standard sample of calcium penicillin was kindly furnished by Dr. Henry Welch, of the Food and Drug Administration.

†These procedures were carried out by Miss Ruth Peterson under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Washington University School of Medicine.

‡These will be reported by Dr. Robert A. Moore, of the Department of Pathology.

and 11). Two patients (Cases 2 and 3) suffered immediate relapses after twenty-four and thirty days of therapy, but both responded well to second courses. In one (Case 2), relapse occurred the day after penicillin was discontinued; in the other, fever began four days after the last dose of the drug (Appendix, Case 3). Two of the patients died in congestive heart failure (Cases 4 and 16). One of these showed no bacteria in sections of the involved heart valve obtained at autopsy (Appendix, Case 4). The other had no clinical evidence of infection at the time of death, but autopsy observations could not be made. The streptococci isolated from two patients (Cases 10 and 14) were relatively resistant to penicillin. One of these patients died, but the other is living and clinically well, although bacteria were still present in the blood at the time the patient left the hospital (Appendix, Case 10).

Staphylococcal Endocarditis.—The six cases of endocarditis caused by staphylococci are summarized in Table IV. An acute and fulminating course was observed in four of the patients (Cases 23, 25, 26, and 28), while a chronic afebrile disease in one (Case 27) was correlated with characteristics of the organism usually associated with lack of pathogenicity.^{7, 8}

Results in this group were excellent; there was only one death and that occurred in the first patient in the entire series (Case 23). It is interesting to note that this patient made a good response to small doses of penicillin; death was due to rupture of a brain abscess.

DISCUSSION

Prior to the use of penicillin, the prognosis of bacterial endocarditis was almost hopeless.⁹⁻¹³ Although the first reports on the use of the drug in the treatment of this disease were discouraging,¹⁴⁻²⁵ many observers have subsequently reported favorable results.²⁶⁻²⁷ Our results likewise confirm the efficacy of penicillin. The magnitude of the advance is strikingly demonstrated in Table V, in which the results in the present series are compared with those obtained in twenty-four patients with streptococcal and staphylococcal endocarditis admitted to the Barnes Hospital over a seven-year period from 1935 to 1941 inclusive.*

Criteria of Cure.—Observations of the natural course of bacterial endocarditis in the years before penicillin therapy have shown that spontaneous remissions occur⁹ and may lead to false conclusions as to the prognosis. In view of this fact, it has been pointed out⁴⁴ that a long follow-up period is necessary in order to demonstrate the value of treatment in this disease. In the present series, it is possible to report that a considerable number of patients are alive and apparently well after periods of from six months to two years since the conclusion of treatment. These observations indicate that beneficial effect is not of a temporary character and increase the probability of actual cure.

In most patients with bacterial endocarditis in whom the infection seems to be controlled with penicillin, it is impossible to observe the microscopic lesions of the valve because the patients are living. In three reported cases^{26, 45} in

*Twenty of these cases were due to *Streptococcus viridans*, three to hemolytic *Staphylococcus aureus*, and one to hemolytic *Staphylococcus albus*.

TABLE IV. TREATMENT OF STAPHYLOCOCCAL

CASE	AGE	SEX	TYPE OF HEART DISEASE	DURATION OF SYMPTOMS	MURMURS*	NUMBER OF POSI- TIVE BLOOD CULTURES BEFORE TREATMENT	MAXIMUM COLONY COUNTS PER C.C.	ORGANISM
23	33	Female	Rheumatic	8 days	M.I., M.S., A.I.	0	1000	Hemolytic Staphylococcus aureus†
24	31	Female	Congenital	5 wk.	I.V.S.	3	200	Hemolytic Staphylococcus aureus†
25	17	Female	Rheumatic	6 days	M.I.	3	160	Hemolytic Staphylococcus aureus†
26	35	Female	None	3 wk.	M.I.	4		Hemolytic Staphylococcus aureus†
27	35	Male	Rheumatic	5 mo.	M.I., M.S.	12	84	Hemolytic Staphylococcus albus§
28	22	Male	None	15 days	M.I.	4		Hemolytic Staphylococcus aureus†

*M.I., Mitral systolic murmur; M.S., mitral diastolic murmur; A.I., aortic diastolic murmur; I.V.S., systolic murmur of patent Interventricular septal defect.

†See appendix.

which the infection was apparently arrested with penicillin, the opportunity to observe sections of the involved valves presented itself when the patients subsequently died of cardiac failure. In two of these cases,⁴⁰ no bacteria were found and evidence of extensive healing was present; in the third,²⁶ although the reparative process was noted, a small nest of bacteria was demonstrated. In one of our patients (Case 4) death from heart failure made it possible to examine a section of the affected valve and no bacteria were seen. Inasmuch as sections of valves in untreated bacterial endocarditis have almost always shown numerous large colonies of bacteria,^{9, 50-52} the foregoing observations constitute strong evidence in favor of the theory that patients with bacterial endocarditis who are clinically well for several months after penicillin treatment are completely cured.

Analysis of Failures.—While a significant number of patients have made an apparent recovery from bacterial endocarditis and while the value of penicillin therapy in this disease seems established, it is desirable to consider carefully those patients in whom the ultimate outcome was unsatisfactory in an attempt to evaluate the factors responsible.

The importance of prompt, intensive, and prolonged penicillin treatment is indicated by the failures recorded in six of our patients who died early in the course of therapy; it is further emphasized when one considers the progressive debility which accompanies systemic infection and the danger of disabling or fatal embolism⁵³ (Appendix, Case 21).

Failure in two of our patients with streptococcal endocarditis (Cases 10 and 14) was adequately explained by the relative resistance to penicillin of the bacteria isolated. It has been shown that certain strains of streptococci are inherently resistant to penicillin^{54, 55}; this is particularly true of *Streptococcus fecalis*,^{54, 56} which causes from 5 to 10 per cent of all cases of streptococcal endocarditis.⁵⁴ In view of these facts, it is clear that at present insensitive bacteria may interfere with recovery in a significant number of patients. In one of our

DOCARDITIS WITH PENICILLIN

RANGE OF DAILY DOSE (UNITS)	ROUTE	DURATION OF TREATMENT (DAYS)	TOTAL PENICILLIN (UNITS)	RESULTS
1,000	I.V.	5	270,000	Died on fifth day of therapy; autopsy revealed rheumatic mitral disease with active vegetations and ruptured brain abscess
0,000	I.V.	13	4,680,000	Well twenty-one months after therapy
0,000-300,000	I.M., I.V.	26	3,120,000	Well twenty months after therapy†
0,000-1,200,000	I.M.	22	11,600,000	Well ten months after therapy
0,000	I.M.	42	20,512,000	Well four months after therapy; one episode of cardiac decompensation
0,000-1,200,000	I.M., I.V.	67	58,640,000	Well one and one-half months after therapy

‡Coagulase positive, mannite fermented.

†Coagulase negative, mannite not fermented.

‡Murmur developed under observation.

patients (Appendix, Case 10) an attempt was made to give enough penicillin to overcome a resistant streptococcus, and a striking decrease in the number of bacteria in the blood was observed. The practical difficulties encountered in the administration of this large amount of penicillin rendered continuation of such therapy impossible, but the results seem encouraging enough to warrant further trials if it becomes feasible to attain sufficiently high levels of penicillin in the body.

In two of our patients (Cases 2 and 3; Appendix, Case 3) relapse occurred, apparently because penicillin was not continued long enough. The histories of several others (Cases 10, 15, 16, 17, 20, and 21) showed that penicillin had been given to them for short periods of time before they came to this hospital and that the clinical manifestations of the disease had recurred shortly after therapy was concluded. In all but one of these patients further prolonged treatment with penicillin resulted in a satisfactory arrest of the infection. In the single unsuccessful instance (Case 10), the evidence points more to inherent resistance of the organism as previously noted. The experience, as a whole, supports the opinion^{26, 29, 46, 57, 58} that penicillin therapy should be continued over a long period of time and does not indicate that the bacteria are likely to acquire enough resistance through one or two inadequate courses of treatment to interfere with subsequent success.

Two patients (Cases 4 and 16) died of cardiac insufficiency and two more (Cases 7 and 27) had episodes of heart failure in spite of the fact that their infections were apparently arrested by penicillin. It has been suggested that cardiac failure may be due to valvular deformity produced by fibrous contractions of healing vegetations,^{46, 49, 58} particularly if the aortic valve is involved.^{34, 49, 58} Although it is not possible to prove that such a mechanism was responsible for heart failure in our patients, it seems probable that ultimate

TABLE V. RESULTS OF THE TREATMENT OF BACTERIAL ENDOCARDITIS BEFORE AND AFTER THE INTRODUCTION OF PENICILLIN*

	ARRESTED	FAILURES	TOTAL
Penicillin treated	18	10	28
Treated before penicillin	0	21	21
Total	18	34	52

The high statistical significance of these figures has been determined by calculation of chi square from a fourfold table utilizing the correction of Yates for small numbers.

survival from the disease must depend as much on the amount of destruction or deformity of the valve leaflets as on the susceptibility to antibacterial action of the organism in the vegetation.

SUMMARY

Twenty-eight patients with bacterial endocarditis due to streptococci or staphylococci were treated with penicillin. The patients were unselected except as to the validity of the diagnosis. The beneficial effect of penicillin in these patients is shown by the following considerations:

1. Clinical arrest has occurred in a significant number.
2. The time elapsing since the completion of therapy indicates that the clinical improvement is not temporary.
3. Histopathologic examination of the valve of one patient dying of heart failure after the completion of treatment revealed no bacteria.

Experience in the treatment of these patients re-emphasized the importance of prompt, intensive, and prolonged chemotherapy.

Relapse occurred in two patients after completion of a first course, but both patients responded to a prolonged second course.

Failure in two patients was explained by penicillin-resistant streptococci

APPENDIX

CASE 3.—This patient, a 26 year old white married housewife,* entered the Barnes Hospital for the first time April 17, 1944. At the age of 7 years she had acute arthritis which affected the joints of the arms and legs. The patient had been in bed at that time for three weeks but had subsequently been in excellent health. In July, 1943, she had an induced abortion followed by rather profuse vaginal discharge. On several occasions dilatation and curettage had been carried out. In February, 1944, the patient was given a sulfonamide drug by a local physician apparently because of the vaginal discharge. She took the drug for several weeks but developed oliguria and was sent to a hospital where she remained for five weeks. There she had temperature elevations as high as 102° F., and two weeks before entry here noted that her fingers were becoming clubbed. She had lost approximately thirty pounds during her illness. Two blood cultures taken in the other hospital were reported positive for *Streptococcus viridans*.

On entry, the temperature was 37.8° C.; pulse, 120; respiration, 20; blood pressure, 100/20/0. The patient was a well developed but poorly nourished white woman who appeared chronically ill. No petechiae were seen. The teeth were carious. The heart was enlarged 11 cm. to the left and 3 cm. to the right. There was a rapid rate with a regular rhythm. At the apex a rumbling diastolic murmur with presystolic accentuation was heard ending in a loud first sound. At the base harsh systolic murmurs and high pitched early blowing diastolic murmurs were heard; the latter were transmitted along the left sternal border. The splenic

*Reported through the courtesy of Dr. Edward Cannady.

CASE 3. (1st. ADMISSION)

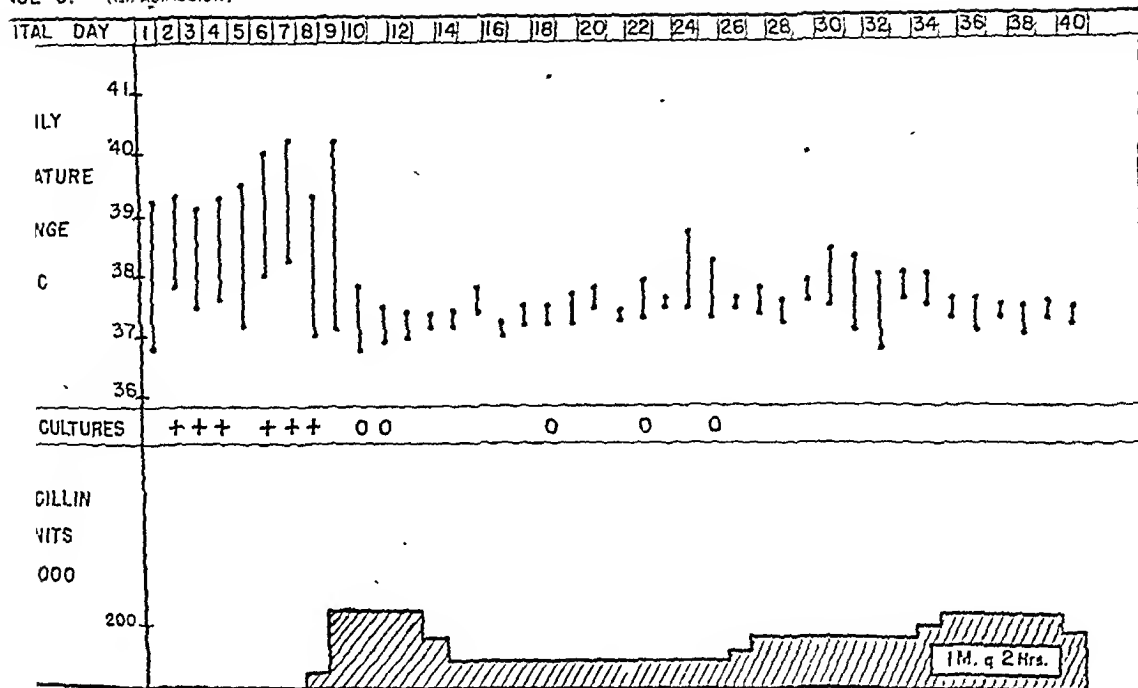


Fig. 1.

CASE 3 (2nd ADMISSION)

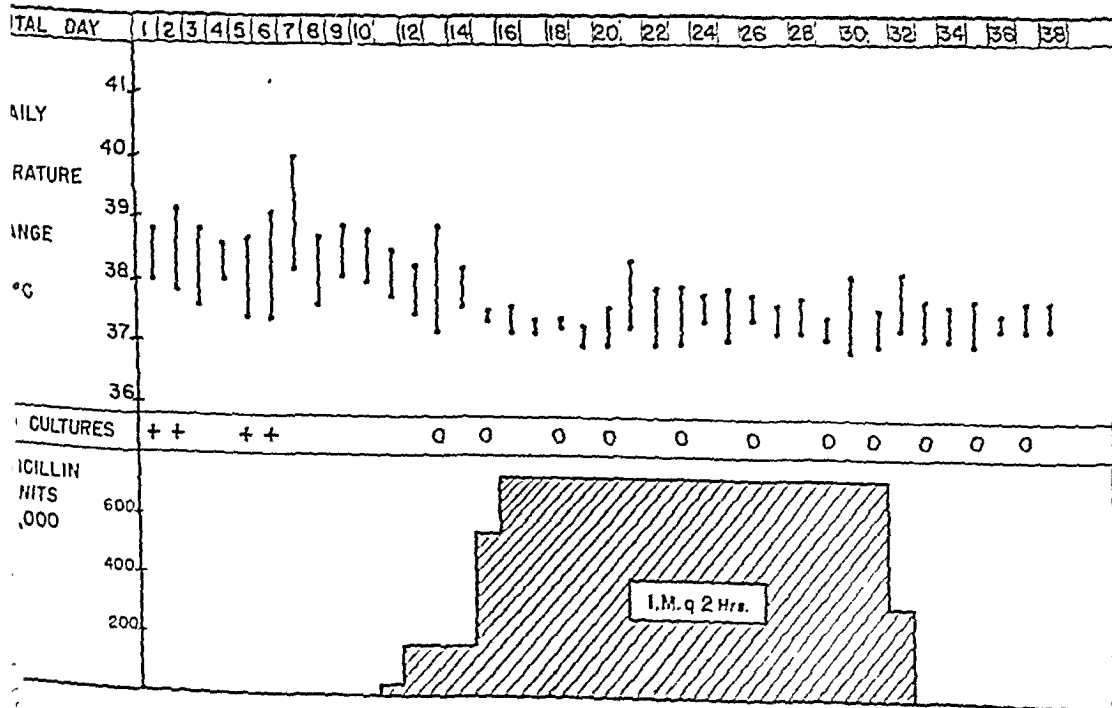


Fig. 2.

tip was just palpable. There was clubbing of the fingers and toes and a suggestive splinter hemorrhage was noted under the nail of the left fifth finger. Pelvic examination was not remarkable except for a creamy vaginal discharge. The remainder of the physical examination was contributory.

The laboratory data revealed that the red blood cell count was 3,600,000 per cubic millimeter with 10 Gm. of hemoglobin per 100 c.c.; the white blood cell count was 8,800, with a normal differential. The urine showed a small amount of albumin but no red cells. Kahn test of the blood was negative. Culture of the vaginal discharge revealed no gonococci. Four consecutive blood cultures were positive for *Streptococcus viridans* with the maximum colony count 320 colonies per cubic centimeter.

Following the report of the positive blood cultures, the administration of 20,000 units of penicillin intramuscularly every two hours was begun (Fig. 1). The temperature, which had been quite septic, fell to within normal limits and remained there throughout the remainder of the hospital course except for occasional elevations above 38° C. The penicillin dosage was lowered to 10,000 units every two hours intramuscularly. Subsequent blood cultures were negative. During the course of penicillin therapy, the patient had repeated episodes of embolic phenomena. The drug was discontinued after thirty days of therapy, at which time the patient had received a total of 4,800,000 units. She left the hospital May 26, 1944, feeling quite well; she was advised to follow her daily temperatures and to report back to the hospital if elevations occurred. On the fourth day after discharge her temperature was noted to be 102° F. and she returned to the hospital for re-evaluation.

Second Admission.—Physical examination revealed the temperature to be 38.2° C.; pulse, 70; respirations, 20; blood pressure, 112/44. The examination was essentially as on the previous admission; no petechiae were noted, but the splenic tip was again palpable.

The laboratory findings revealed a persistent anemia and a normal white blood cell count. The urinary sediment showed from 2 to 3 red cells per high-powered field. Three consecutive blood cultures were again positive for *Streptococcus viridans*. Penicillin therapy was instituted with dosages of 20,000 units intramuscularly every two hours (Fig. 2).

The temperature which had ranged between 38° C. and 39° C. for the most part fell to within normal limits within twenty-four hours of the reinstitution of penicillin therapy. The dosage was increased to 60,000 units every two hours intramuscularly and continued at that level for twenty-two days when it was discontinued. At that time the total penicillin given was 12,780,000 units. Subsequent blood cultures were negative; the patient's course was uneventful, and she was discharged afebrile and asymptomatic July 8, 1944. On April 3, 1945, she re-entered for the third time to receive prophylactic penicillin therapy for dental extraction. Her general condition was good, blood cultures were negative, and her hospital course was uneventful. The patient has remained very well.

Summary.—A 26-year-old woman who had rheumatic fever at the age of 7 years entered the hospital with subacute bacterial endocarditis due to *Streptococcus viridans*. She received penicillin for thirty days and made a good clinical response, only to relapse four days after discharge. She was then given a second, more intensive course of penicillin, responded well, and has remained in good health since discharge nineteen months ago.

CASE 4.—This patient, a 56-year-old single white man, entered the Barnes Hospital for the first time July 3, 1944, complaining of pain in the left heel and calf for one month and a pruritic rash for three days. At the age of 21 years he had had migratory joint pains and the following year a similar attack. Six months before entry the patient had had oral manipulations apparently designed to make his dentures fit better. One month before entry he developed pain in the left heel which radiated up the calf of the leg and which was accompanied by swelling of the ankles and knees. He then noted palpitation, dyspnea on exertion, and increasing fatigue and consulted a local physician who told him he had "arthritis" and treated him with "arthritis shots." Four days before entry the patient was given sulfathiazole by his physician and on the day before entry he developed a red, itching, maculopapular rash which was generalized.

Physical examination showed a temperature of 38° C.; pulse, 140; respirations, 36; blood pressure, 110/60. The patient was well developed and well nourished and appeared acutely ill. He was slightly cyanotic and markedly short of breath. There was a generalized maculopapular erythematous rash involving the extremities and the trunk, but no petechiae were seen. The neck veins were distended. Bilateral moist basal râles were heard at the lung bases. There were dullness, decreased tactile fremitus, and diminished breath sounds at the right base. The heart was slightly enlarged to the left with a rapid, regular rhythm and sounds of poor quality. Ocular and carotid sinus pressure did not affect the heart rate. Subsequently, when the rate was slowed, a harsh systolic murmur could be heard at the apex where there was also a low-pitched diastolic rumble; a high-pitched early diastolic murmur was heard along the left sternal border. Examination of the abdomen revealed the liver edge 6 cm. below the costal margin. No other organs or masses were palpated. The remainder of the physical examination was noncontributory.

The laboratory studies showed that the red blood cell count was 4,100,000 with 12 Gm. of hemoglobin; the white blood cell count was 8,900 with a normal differential. Examination of the urine showed no albumin or red cells. The blood Kahn reaction was negative. The blood sulfathiazole level was 0.3 mg. per cent. The venous pressure was 150 mm. of saline; the decholin circulation time, 21 seconds. An electrocardiogram on entry showed auricular flutter with two-one block.

ASE 4.

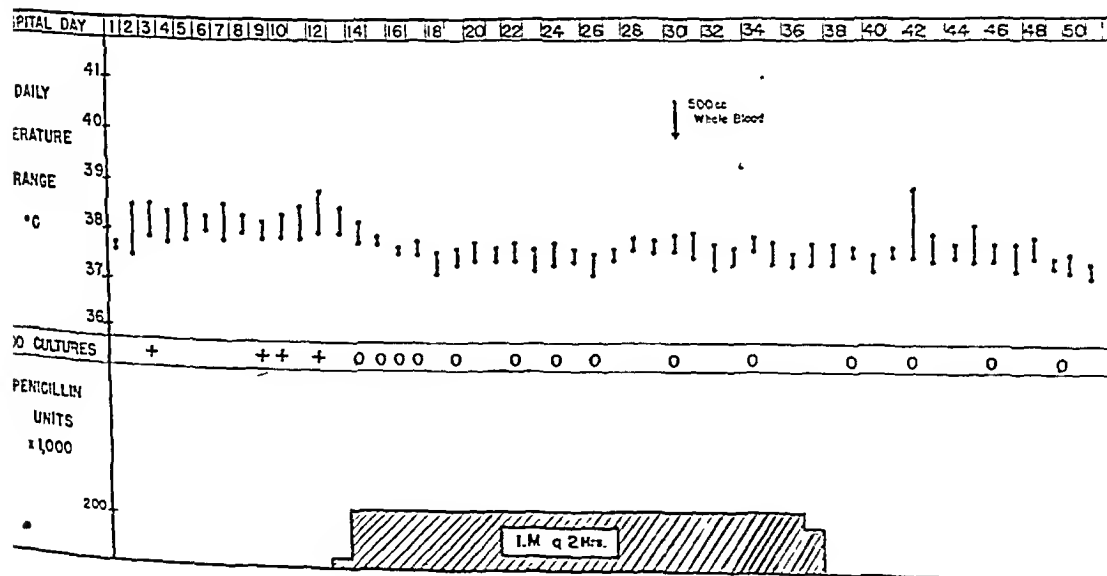


Fig. 3.

The patient was digitalized, and after having transitory auricular fibrillation, the heart reverted to a normal rhythm. Because of continuing low-grade fever, blood cultures were drawn and the first four were positive for *Streptococcus viridans*. The diagnosis of subacute bacterial endocarditis was thus confirmed and the administration of 20,000 units of penicillin intramuscularly every two hours was begun (Fig. 3). There was a good clinical response and all subsequent blood cultures were negative. After twenty-five days of therapy, during which the patient received 5,980,000 units of penicillin, the drug was discontinued. Four days later, the patient complained of pain in the hand, and, although no lesions developed, it was thought that perhaps he had had an embolism. He subsequently was asymptomatic except for marked limitation of his cardiac reserve and he was discharged from the hospital Aug. 23, 1944. He returned to the hospital in one week because of cardiac decompensation but had no symptoms

to suggest recurrence of bacterial endocarditis; repeated blood cultures were negative. He failed to respond to cardiac therapy, however, and gradually failed. He expired Sept. 20, 1944, in an attack of acute pulmonary edema.

At post-mortem examination the heart was found to be very large; the aortic valve was grossly deformed and showed large fungating lesions attached to the valve leaflets. Part of this mass was macerated aseptically and cultured, but no organisms were recovered. The Department of Pathology also cultured the valve, and on the second subculture a growth of *Streptococcus viridans* was obtained. Microscopic examination of the valve, however, showed healing, and bacterial stains of sections of the vegetation and valve showed no organisms.

Summary.—A 56 year-old man who had two bouts of rheumatic fever was admitted to the hospital with subacute bacterial endocarditis due to *Streptococcus viridans*. He received a course of penicillin and did well except for a limited cardiac reserve. One month later he died of intractable heart failure without clinical, laboratory, or pathologic evidence of recurrence of the endocarditis.

CASE 6.—This patient, a 22 year old single white man, was admitted to the Barnes Hospital for the first time Sept. 25, 1944, complaining of weakness and fatigue for seven weeks and afternoon fever for five weeks. At the age of six he had had scarlet fever which left him with a "bad heart." He had been frail and thin ever since, but had apparently gotten along fairly well until two months before entry when he began to notice increasing fatigue and pains in the arms and chest. Five weeks before entry he had a tooth extracted and one week later developed red spots on the finger tips. For about one month he had been short of breath and for two weeks he had had ankle edema. For four days before entry he was given a sulfonamide drug.

Physical examination showed a temperature of 37.8° C.; pulse, 94; respirations, 18; blood pressure, 114/58. The patient was a well developed, extremely thin young male who looked chronically ill. Petechiae were noted on the palms, finger tips, and dorsum of the feet. The neck veins were not distended. The lungs were clear. The heart was markedly enlarged both to the right and to the left. The rhythm was regular. A loud harsh systolic murmur was heard at the apex where there was also a low pitched diastolic rumble. The splenic tip could be felt from 4 to 6 cm. below the costal margin. No other abdominal masses were palpable and the remainder of the examination was noncontributory.

The laboratory findings showed that the red blood cell count was 1,320,000; hemoglobin, 12.5 Gm; white cell count, 9,500; the differential count revealed a shift to the left. The urine showed a small amount of albumin and an occasional red cell. The blood Kahn was positive. The first three blood cultures were negative and the fourth was positive for alpha hemolytic streptococci. Four subsequent blood cultures were likewise positive for alpha hemolytic streptococci.

On the basis of these results the administration of penicillin, 40,000 units intramuscularly every two hours, was started (Fig. 4). The temperature, which had been septic in type until the institution of penicillin therapy, fell to within normal limits thereafter. Penicillin was continued for nineteen days, during which time the patient received 3,850,000 units. Several days before it was discontinued one petechia was noted on the left hand. Subsequent blood cultures were all negative. The patient, as noted, had a positive Kahn test but denied exposure to syphilis; the quantitative Kahn was 40 units. It was felt that nothing more would be done at that time in regard to the question of possible syphilis. The patient left the hospital Nov. 11, 1944.

He did very well and subsequently returned one year later for reevaluation. At that time physical examination was as before, except that there were no petechiae and the patient had developed an early high pitched diastolic murmur, heard at the aortic area and along the left sternal border. Repeated blood cultures were negative on this admission, as were the Kahn test and a lumbar puncture.

Summary.—A 22 year old man who had scarlet fever apparently followed by acute rheumatic fever at the age of 6 years entered the hospital because of subacute bacterial endo-

carditis due to *Streptococcus viridans*. He was treated with intramuscular injections of 40,000 units of penicillin every two hours for nineteen days and made a good recovery. He has remained well for fourteen months and is an example of the successful treatment of streptococcal endocarditis with penicillin. The development of aortic incompetency suggests that the aortic valve was the seat of vegetation and, in healing, had become more deformed.

CASE 6

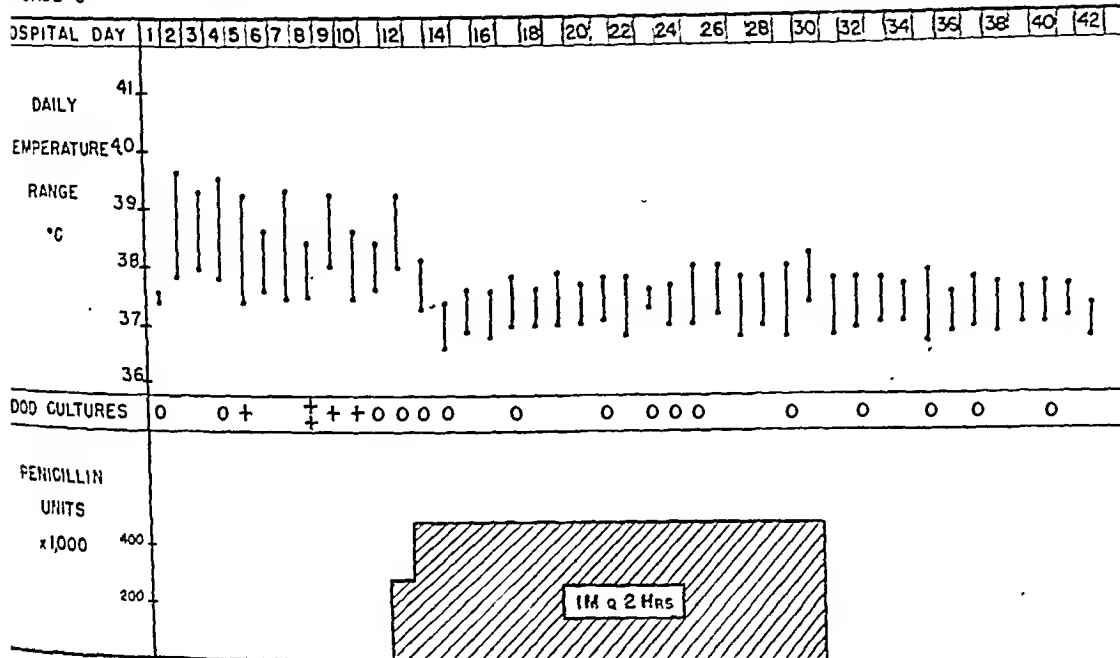


Fig. 4.

CASE 10.*—This patient, a 24-year-old white married housewife, entered the Barnes Hospital for the first time March 12, 1945, complaining of fever, joint pains, and an erythematous rash. The patient had always enjoyed good health and had never had acute rheumatic fever or chorea. About Dec. 1, 1944, three months prior to entry, she had a miscarriage. The fetus was said to have been approximately 6 weeks old, and subsequent to its passage the patient had vaginal bleeding in decreasing amounts for one month; she felt well otherwise until Jan. 1, 1945, when she noted the onset of malaise, weakness, and afternoon fever ranging from 100 to 101° F. She was given a sulfonamide drug, but her symptoms did not improve, and dilatation and curettage were carried out. Subsequent to this procedure the patient was discharged from the hospital in which the operation had been performed, but nine days later she re-entered there and it was found that she had a heart murmur which had not been previously noted. The sulfonamide therapy was continued and the patient was given 10,000 units of penicillin intramuscularly every two hours for nine days. On receiving penicillin, her symptoms cleared, but after the drug was discontinued the temperature rose again and penicillin was reinstituted. Shortly before admission to the Barnes Hospital, the dose was increased to 20,000 units every two hours. During the period that the patient had not been receiving penicillin she had noticed petechiae in the skin and several small splinter hemorrhages under the fingernails. Likewise, when off penicillin she had had some generalized aches and pains which had abated during therapy. The patient had had numerous night sweats, but during the period she had received penicillin, these had decreased. During her illness she had lost about ten pounds.

*Reported through the courtesy of Dr. C. N. Duden.

Physical examination on entry showed a temperature of 37° C.; pulse, 100; respirations, 20; and blood pressure, 110/70. The patient was a well-developed but undernourished female lying quietly in bed in no apparent discomfort. The skin was pale and several petechiae were seen on the third finger of the right hand. The teeth and gums appeared normal except for several petechiae opposite the second right lower molar. The lungs were clear. The heart was enlarged, with the left border of dullness in the fifth interspace, 10.5 cm. from the mid-sternal line. There was no thrill, but a high-pitched grade 2 systolic murmur could be heard at the apex and was transmitted over the entire precordium. The first sound at the mitral area and the second pulmonary sound were not accentuated. The spleen was not palpable. Pelvic examination was negative. Examination of the extremities revealed marked clubbing of both fingers and toes.

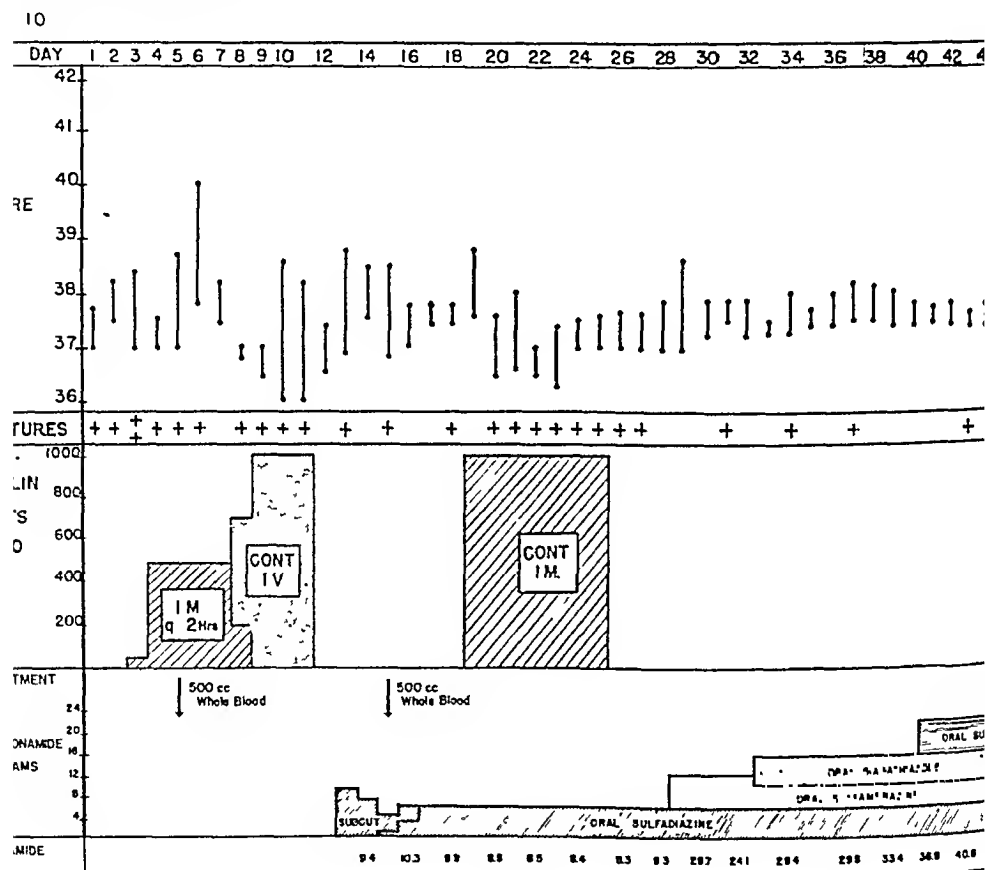


Fig. 5.

The laboratory data showed that the red blood cell count was 4,100,000 with 8 Gm. of hemoglobin. The white blood cell count was 5,600, and the differential count showed a slight left shift. Examination of the urine revealed a small amount of albumin and many red cells. The stool gave a faintly positive test for occult blood. The blood Kahn reaction was negative. The sedimentation rate was 0.2 mm. per minute. Four blood cultures, drawn within the first three hospital days, were all positive for *Streptococcus viridans*, with colony counts ranging from 26 to 170 colonies per cubic centimeter. An electrocardiogram showed only sinus tachycardia. Blood counts were repeated at very frequent intervals and showed a persistent anemia, a normal to slightly elevated white cell count, and often a slight left shift in

the differential count. The urine was free of albumin and rarely showed more than a few red cells. Many urinary sediments showed no red cells at all.

On March 14, 1945, the third hospital day, administration of penicillin, 40,000 units intramuscularly every two hours, was started (Fig. 5). This dose was continued for a little over four days, during which time blood cultures were consistently positive for *Streptococcus viridans* with colony counts as high as 50 or 60 organisms per cubic centimeter. During this period the patient continued to have emboli and her temperature, except for one elevation following transfusion, was of a low-grade variety. On March 19, intravenous penicillin, 1,000,000 units per twenty-four hours, was begun. Despite this increase in dosage, the blood cultures continued to be positive and there were repeated emboli. The route of administration was changed to a continuous intramuscular drip, and although the colony count decreased somewhat, blood cultures were consistently positive. On April 5, 1945, penicillin was stopped. In vitro tests showed this streptococcus to be relatively resistant to penicillin; inhibition of growth was never obtained with less than 3.5 units per cubic centimeter.*

Sulfadiazine, which had been started March 24, was continued, and April 9 sulfamerazine was added in full dosage, blood sulfonamide levels of the order of 25 mg. per cent being obtained. On this regimen the patient's condition remained approximately the same. Blood cultures continued to be positive. On April 13, sulfathiazole in full dosage was added to the list of medications and the total sulfonamide blood level rose to about 30 mg. per cent. On April 22, sulfapyridine likewise was given, so that the patient was receiving four sulfonamides; the combined blood level reached 40 mg. per cent. The patient continued to have a low-grade fever, positive blood cultures, and occasional emboli. At least once there was evidence of cerebral embolization. The sulfonamide blood level got as high as 50 mg. per cent without evidence of renal damage other than that present before any of the drugs were given.^{59, 60} Sulfonamide medication was discontinued, however, May 10 because there was no indication of any beneficial effect.

On July 26, penicillin therapy was reinstituted in massive doses by the constant intravenous route, the patient receiving 4,000,000 units every twenty-four hours (Fig. 6). Previous work² had suggested that such amounts would be necessary in order to reach blood levels capable of affecting an organism resistant to the degree of this one. Prior to the reinstitution of penicillin, colony counts ranged from 50 to 100 per cubic centimeter of blood. With penicillin therapy, they rapidly fell to an average of about 1 colony per cubic centimeter of blood. Finally, beginning Aug. 1, 1945, the sixth day after the massive doses had been started, blood cultures were entirely negative on three occasions. They then became positive again, although usually only one of the broths, or perhaps one pour plate, would show a colony. Great difficulty was experienced in maintaining the continuous intravenous infusion so the route was changed to hourly intramuscular injections of from 150,000 to 200,000 units. However, this frequent intermittent therapy had to be stopped because of pain, low-grade fever, and signs of inflammation at the sites of injection. An attempt at continuous intrasternal infusion† was unsuccessful because flow could not be maintained. Therefore, the continuous intravenous method was reinstituted and 4,000,000 units of penicillin and 50 mg. of heparin were given in 2,000 c.c. of saline every twenty-four hours.

On the fifth day of this regimen, the patient developed a high septic fever with ranges of temperature from 37.2 to 42° C. During this period the urinary findings were unchanged; blood cultures showed no increase in *Streptococcus viridans*, although three, in addition, showed *Chromobacterium prodigiosum*. The patient's condition rapidly became critical. She was given plasma, sulfadiazine, and sulfamerazine, was digitalized with intravenous lanatoside C; heparin was discontinued. Little change was noted until penicillin was stopped; within six hours the high fever subsided and the patient improved remarkably. It was thought at the time that this stormy period had been a manifestation of sensitivity to penicillin and in

*The organism was also found to have a high in vitro resistance to sulfonamide drugs and to streptomycin.

†Done by Dr. Carl V. Moore.

an attempt to confirm this, intravenous penicillin was again begun Oct. 8, 1945 (Fig. 6). The patient was given 5,000,000 units for two and one-half days, but during this period she developed no fever nor any other signs of toxicity. The drug was not continued because further veins could not be reached, even with surgical intervention. Continuous intramuscular injection could not be done because of local reactions, and it was not feasible to give enough orally to effect the organism.

During the last two months in the hospital, the patient appeared to improve despite the fact that she still had positive blood cultures. She was finally discharged, ambulatory, Oct. 22, 1945. During her hospital stay she had received seven blood transfusions, a high calorie diet, and added vitamins.

Three months after discharge, the patient had gained weight, was maintaining essentially normal activity, and was feeling well.

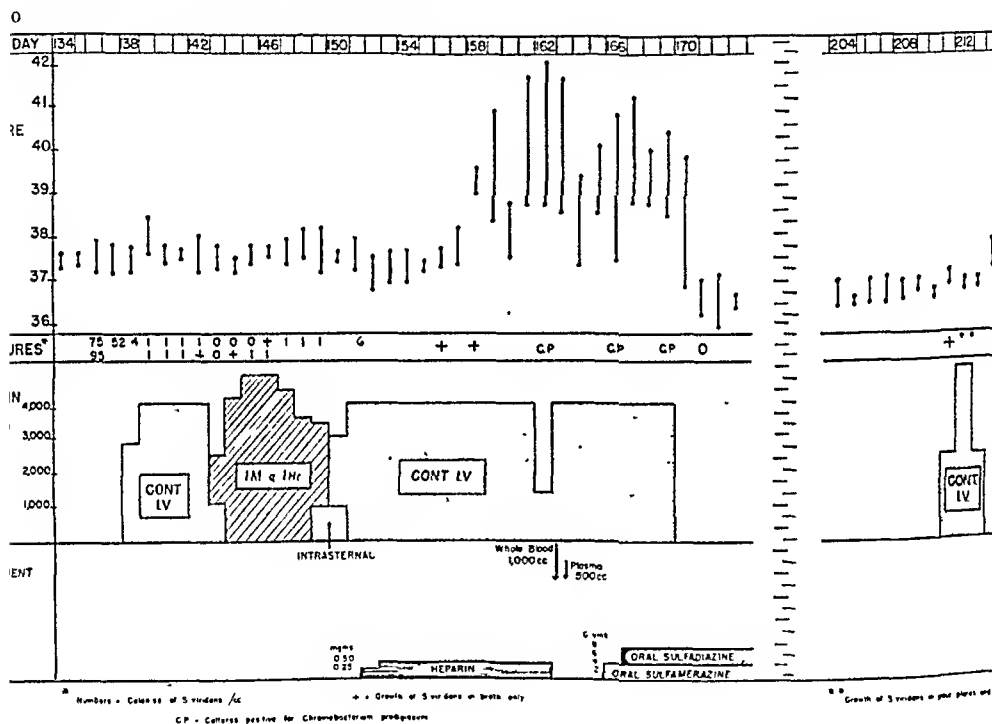


Fig. 6.

Summary.—A 24 year-old woman without antecedent history of rheumatic fever or heart disease was admitted to the hospital with subacute bacterial endocarditis due to *Streptococcus viridans*. Following lack of response to usual doses of penicillin and to simultaneous therapy with four sulfonamides, she was given massive doses of penicillin. Although the bacteremia became less intense, it was not controlled, and the patient was discharged improved but with blood cultures still positive.

CASE 21.—This patient, a 22-year-old white married woman, entered the Barnes Hospital Aug. 30, 1945, complaining of fever, anemia, weight loss, and pain in the back and legs. The patient gave no past history of definite acute rheumatic fever, although in her youth she had had episodes of pain in her legs. Until the onset of the present illness she had been entirely unaware of any cardiac disease. She had been apparently well until the summer of 1944, at which time she had had a tooth extracted; an abscess developed at the site of extraction and the patient felt poorly for several months, although she continued to be up

and about and took care of her household duties. Ten months before entry she developed a swelling in the tip of her left index finger, and a similar lesion appeared in the right large toe. Both sites were very painful to touch but subsided in several weeks. In February, 1945, the patient had a "nervous breakdown," during which time she was apparently depressed. In

CASE 21

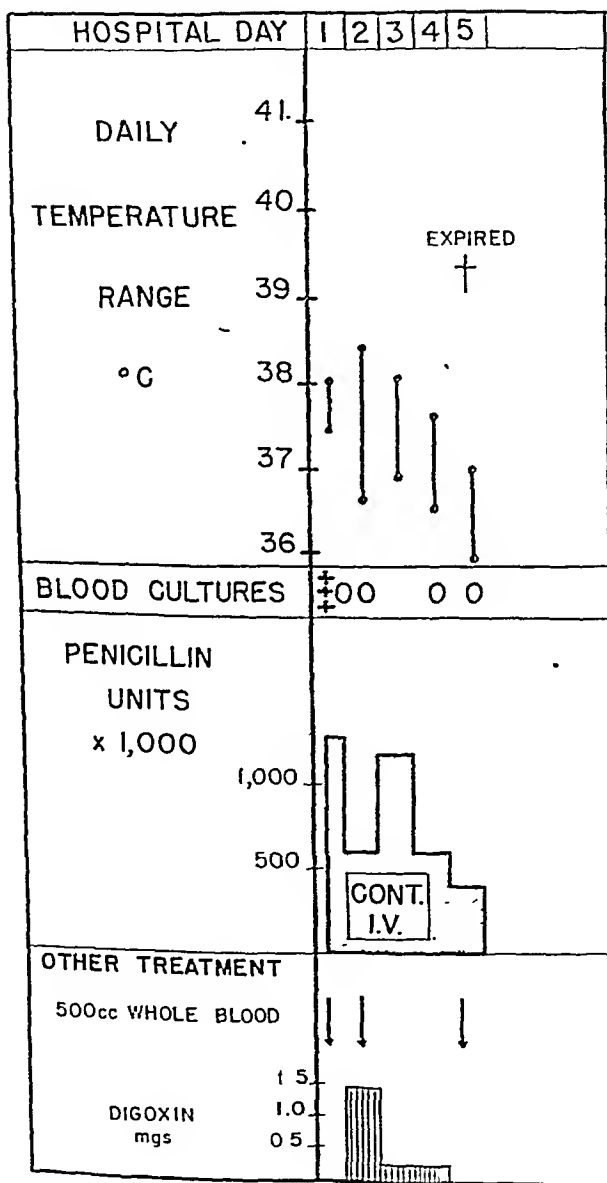


Fig. 7.

March, 1945, she was told by her local physician that she was anemic; she felt weak and became progressively more tired. Because of the development of severe pain in both flanks, she finally went to a hospital where she was told she had an infected kidney and a leaky heart. She was given penicillin for three days and then discharged. From that time on she became gradually

worse, and for a month before entry had not seen any doctor because of discouragement with previous treatment. For several days before entry here she had severe pain in both legs, worse in the right.

Physical examination on admission showed a temperature of 38° C.; pulse, 120; respiration, 28; blood pressure, 92/60. The patient appeared in extremis. The skin was dry, there was marked wasting, and great numbers of petechiae were seen over the extremities in the trunk. Many petechiae were observed in the conjunctivae and the sclerae, and numerous fresh hemorrhages were seen in the optic fundi. The mucous membranes of the mouth likewise showed petechiae. The neck was not stiff. The posterior cervical lymph nodes were enlarged. Examination of the chest revealed medium and fine inspiratory rales over the left lower lobe, but the remainder of the chest was clear and there was no friction rub. The heart was enlarged with a diffuse apical impulse in the sixth interspace outside the mid-clavicular line. A presystolic thrill was felt at the apex. The sounds were rapid and of poor quality except that the second pulmonic sound was accentuated. A harsh, loud systolic murmur could be heard at the apex and was transmitted to the axilla and over the entire precordium. There was also a rumbling mid-diastolic murmur at the apex. The abdomen was tense, but the liver edge could be felt 6 cm. below the costal margin and was slightly tender. The spleen was not palpable. There was bilateral costovertebral angle tenderness. The fingers were clubbed. Bilateral ankle edema was noted. The right foot was cold and the toes were blue. Both femoral arteries pulsated well, but no popliteal or dorsalis pulsations could be felt on the right.

The laboratory data showed that the red blood cell count was 2,800,000 with 7.5 Gm. of hemoglobin. The white cell count was 31,000, with a moderate left shift in the differential. The urine showed a moderate amount of albumin and the sediment contained from one to two red cell casts, 30 to 40 white cells, and 10 red cells per high-power field. The Kahn test was negative. Three blood cultures taken at ten-minute intervals shortly after admission were all positive for *Streptococcus viridans*. The nonprotein nitrogen was 36 mg. per cent; the total proteins, 4.8 Gm., with 2.5 Gm. of albumin and 2.3 Gm. of globulin. An electrocardiogram showed myocardial damage and sinus tachycardia. Following digitalization with intravenous digoxin there was partial heart block with prolonged A-V conduction, frequent blocked ventricular beats, and excess digitalis effect. Two sedimentation rates were within normal limits.

Immediately after the blood cultures were obtained, intravenous penicillin was begun, and during the first hour the patient received 150,000 units (Fig. 7). A continuous intravenous drip was maintained, and she received approximately 1,000,000 units of the drug daily. Within eleven hours after the institution of penicillin therapy the blood culture was negative, and during the remaining four days of life four other blood cultures were negative. The white count remained elevated. The venous pressure stayed within normal limits until the third of three blood transfusions was being given, at which time it rose and the transfusion had to be discontinued. Despite the intensive therapy the patient developed new petechiae and a left pleural friction rub was noted. She became stuporous and then comatose and died on the morning of Sept. 3, 1945. Her temperature never rose above 38.4° C. and terminally fell to a subnormal level. At the time of death she had received 4,150,000 units of penicillin.

Summary.—A 22-year-old woman entered the hospital in extremis with overwhelming subacute bacterial endocarditis due to *Streptococcus viridans* and recent arterial embolization. Despite penicillin therapy which sterilized the blood stream within eleven hours, the patient died on the fourth hospital day.

CASE 25.*—This patient, a 17-year-old white single schoolgirl, was admitted to the Barnes Hospital for the first time March 23, 1944, because of chills and fever of six days' duration. At the age of 11 years she had had acute rheumatic fever and since that time had been known to have a heart murmur. She had been quite well, however, until one month before entry, when her left ear began to drain. Aside from two transient episodes of pain

*Reported through the courtesy of Dr. H. L. Alexander.

in the ear two weeks before entry, she had had no other symptoms. Six days before admission she had several shaking chills and her temperature rose to 105° F. She complained of pain in the right hip and in the fourth finger of the left hand and felt weak and exhausted. She was admitted to a hospital where two blood cultures were said to show "diplococci resembling pneumococci." She was given sulfadiazine and three blood transfusions but continued to have a high septic fever and to do poorly. She was transferred to the Barnes Hospital for further treatment.

Physical examination showed a temperature of 40.8° C.; pulse, 122; respiration, 26; and blood pressure, 130/64. The patient was a well-developed, well-nourished young woman who appeared acutely and critically ill. She complained only of feeling "chilly." Her skin was warm and moist; several petechiae were present on the hands and feet. Bloody crusts were

CASE 25

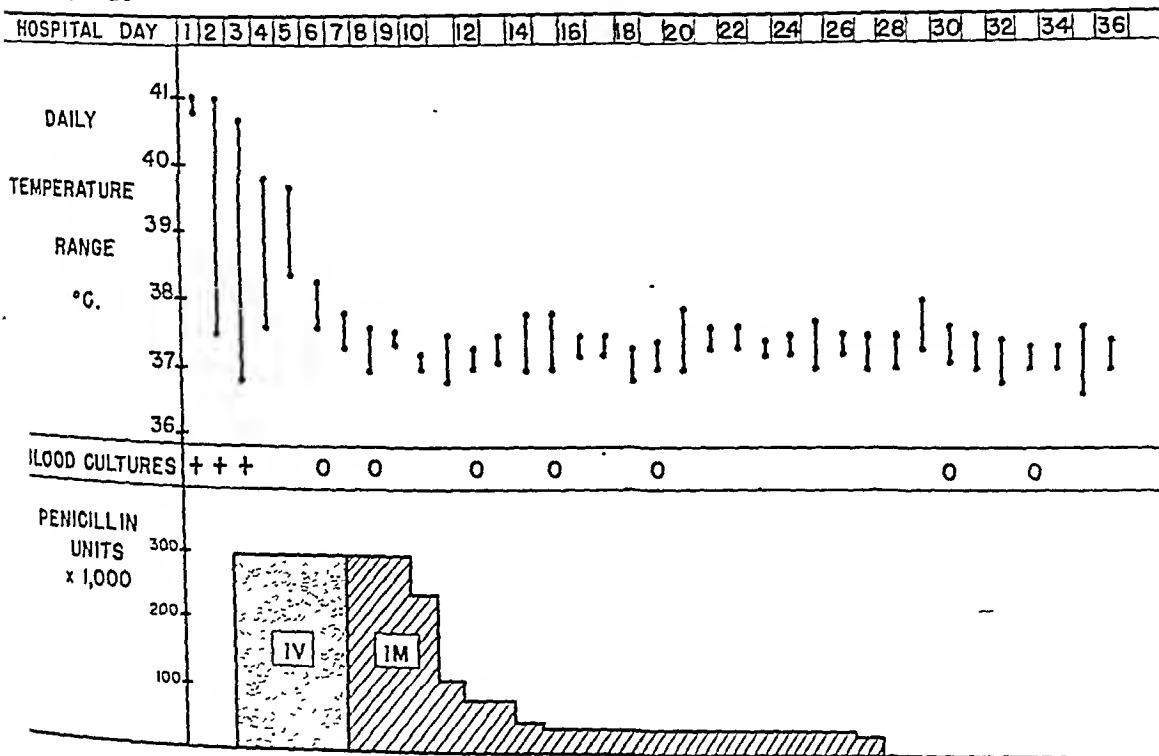


Fig. 8.

noted in both nostrils. The lungs were clear. The heart was slightly enlarged to the left in the fifth interspace; the rate was rapid and the rhythm was regular. The second pulmonic sound was greater than the second aortic sound. A rough high-pitched systolic murmur was heard maximally at the apex, but it was transmitted over all of the precordium. The tip of the spleen was just barely palpable at the costal margin. No other organs or masses were felt in the abdomen. The remainder of the physical examination was not remarkable.

Laboratory data revealed that the red blood cell count was 4,400,000 with 15 Gm. of hemoglobin. The white cell count was 10,500, with a marked left shift. The urine showed no albumin or red cells. The blood Kahn test was negative. Two blood cultures taken on entry were both positive for hemolytic *Staphylococcus aureus*, the maximal colony count being 140 per cubic centimeter. The organism fermented mannite and was coagulase positive.

On the evening of the second hospital day, penicillin was begun in doses of 12,500 units every hour; the drug was injected into the tubing of a continuous intravenous drip (Fig. 8).

On the following day the dose was changed to 25,000 units every two hours and this regimen was continued for five days. The temperature, which had shown marked swings from 37.5 to 41° C., began to respond within forty-eight hours of the institution of penicillin therapy and by the fifth day had fallen to normal limits. At that time administration was changed to the intramuscular route and all subsequent penicillin was given this way. During the first two or three hospital days the patient continued to have embolic phenomena and an occasional red cell was found in the urinary sediment, but otherwise her hospital course was very uneventful. The temperature remained normal and the penicillin dose was gradually decreased so that in the last four days she received only 5,000 units every three hours. All blood cultures taken after institution of penicillin therapy were negative and the patient left the hospital April 28, 1944. She has been exceedingly well in the twenty-one months since discharge.

Summary.—A 17-year-old girl with known rheumatic heart disease of six years' duration was admitted to the hospital with acute bacterial endocarditis due to hemolytic *Staphylococcus aureus*. She was treated with relatively small doses of penicillin for twenty-six days and responded dramatically. She has remained well since discharge, twenty-one months ago.

Acknowledgment is made to Miss Brigitte Watty for preparation of the charts.

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MAINTENANCE OF THERAPEUTIC BLOOD CONCENTRATIONS OF PENICILLIN FOR TWENTY-FOUR HOURS FOLLOWING SINGLE INJECTIONS OF PENICILLIN-BEESWAX-PEANUT OIL MIXTURES

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I NTRAMUSCULAR injection of penicillin-beeswax-peanut oil mixtures is one of the most promising methods so far described for prolongation of the action of penicillin in the body.¹ Optimal results are obtained with mixtures containing approximately 5 per cent beeswax and 300,000 units of penicillin per cubic centimeter.² Such mixtures are now available commercially.

Ideally, it should be possible to administer an average day's supply of penicillin in a single injection of a penicillin-beeswax-peanut oil mixture, with maintenance of effective therapeutic concentrations in the blood stream for a period of twenty-four hours. Unfortunately, this objective has not been attained with single intramuscular injections of 300,000 units (1 c.c.) of penicillin in beeswax-peanut oil.² In a series of patients studied in this hospital, assayable levels were present for from eight to twenty-eight hours, but in approximately two-thirds of the individuals penicillin could be detected for no longer than twelve hours.³ The results were considerably better when the material was injected subcutaneously rather than intramuscularly, assayable levels then being present for twenty hours in two-thirds of twenty-five patients.³

The purpose of the present report is to describe the results of assays of the blood performed every four hours for thirty-six hours following single intramuscular and subcutaneous injections of 600,000 units (2 c.c.) of a commercially prepared penicillin-beeswax-peanut oil mixture. After warming the mixture in the incubator at 37° C. for from sixty to ninety minutes, 2.2 c.c. were aspirated into a 5 c.c. Luer-Lok syringe through a No. 15 needle; a No. 17 needle was used for the injection into the tissues. Intramuscular injections were made in the upper outer quadrant of the buttock. Subcutaneous injections were made either over the insertion of the deltoid muscle, or along the lateral aspect of the thigh, overlying the fascia lata. The individuals used for the study were all healthy young men suffering from acute gonococcic urethritis; twenty patients received the mixture intramuscularly and twenty subcutaneously (eleven over the deltoid muscle and nine along the lateral aspect of the thigh). As in the earlier studies, moderate soreness was noted at the site of the injection for from twenty-four to forty-eight hours and was less severe following subcutaneous than following intramuscular injections. No other local or systemic manifestations of toxicity were observed, and it may especially be noted that with the subcutaneous route no induration, nodule formation, or sterile abscesses occurred. It should be

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emphasized, however, that the mixture must be injected well down into the subcutaneous tissue, since absorption is impaired and nodules may develop if the material is inserted into the skin itself. Penicillin assays were performed by the method of Kirby and Rantz.¹

Results of the assays are presented in Tables I and II. Only the levels obtained from twelve to thirty-six hours after the injections are included; in about one-half of the patients assays were also made at one-half, one, four, and

TABLE I. BLOOD LEVELS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) IN TWENTY PATIENTS FOLLOWING SINGLE INTRAMUSCULAR INJECTIONS OF 600,000 UNITS (2 c.c.) OF A PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

CASE	HOURS							TOTAL DURATION
	12	16	20	24	28	32	36	
1	0.2	0.1	0	0	0	0	0	16
2	0	.05	0	0	0	0	0	16
3	0.1	0.1	0.1	0	0	0	0	20
4	0.2	0.2	0.1	0	0	0	0	20
5	.1	.2	.1	0	0	0	0	20
6	.2	.4	.1	0	0	0	0	20
7	.4	.1	.1	0	0	0	0	20
8	.1	.1	.07	0	0	0	0	20
9	1.0	.2	.2	0	0	0	0	20
10	.2	.2	.2	.1	0	0	0	24
11	0.1	.07	.2	.1	0	0	0	24
12	.07	.2	.07	.2	0	0	0	24
13	.2	.07	.07	.05	0	0	0	24
14	0.1	.2	.05	.2	0	0	0	24
15	.1	.2	.7	.07	0	0	0	24
16	.2	.1	.1	.07	0	0	0	24
17	.2	.2	.2	.2	0	0	0	24
18	.4	.2	.1	.2	.07	0	0	28
19	.4	.2	.1	.1	0	.04	0	32
20	.2	.2	.2	.2	.1	.2	.1	36

TABLE II. BLOOD LEVELS OF PENICILLIN (UNITS PER CUBIC CENTIMETER) IN TWENTY PATIENTS FOLLOWING SINGLE SUBCUTANEOUS INJECTIONS OF 600,000 UNITS (2 c.c.) OF A PENICILLIN-BEESWAX-PEANUT OIL MIXTURE

CASE (ARM OR THIGH)	SITE	HOURS							TOTAL DURATION
		12	16	20	24	28	32	36	
1	A	.5	.2	.1	0	0	0	0	20
2	A	.2	.2	.1	0	0	0	0	20
3	A	.2	.1	.07	0	0	0	0	20
4	A	.2	.4	.2	.05	0	0	0	24
5	A	.2	.2	.1	.1	0	0	0	24
6	T	.5	.2	.1	.1	0	0	0	24
7	T	.2	.2	.1	.1	0	0	0	24
8	A	.1	.7	.1	.05	.07	0	0	28
9	A	.2	.2	.7	.2	.1	0	0	28
10	A	.2	.2	.4	.2	.04	0	0	28
11	T	.1	.1	.07	.1	.07	0	0	28
12	T	.7	.2	.5	.1	.1	0	0	28
13	A	.1	.04	.2	.05	0	.1	0	32
14	A	.2	.2	.2	.1	.07	.04	0	32
15	T	.5	.1	.1	.1	.2	.05	0	32
16	T	.7	.2	.5	.2	.07	.1	0	32
17	A	.2	.2	.07	0	.07	0	.04	36
18	T	.7	.2	.4	.1	.1	.1	.05	36
19	T	.7	.2	.2	.2	.2	0	.04	36
20	T	.2	.2	.5	.2	.2	.2	.2	36

eight hours, with results similar to those obtained in the earlier studies with 300,000 units. Measurable concentrations of penicillin were present for from sixteen to thirty-six hours following intramuscular injection and for from twenty to thirty-six hours following subcutaneous administration. The results following subcutaneous administration were considerably better, from the standpoint of prolongation of assayable blood levels, than were those following intramuscular administration. Following intragluteal injections, nine patients had measurable concentrations for no longer than twenty hours, and in two of these the duration was only sixteen hours. With the subcutaneous route, on the other hand, assayable levels were present for less than twenty-four hours on only three occasions, and in none of these was the duration less than twenty hours. In other words, with the subcutaneous route, measurable levels were present for twenty-four hours or longer in 85 per cent of the patients, while with the intramuscular route twenty-four-hour levels were obtained in only a little over one-half of the patients, or 55 per cent. The results with subcutaneous administration appeared equally as good, and possibly a little better, when the material was injected along the lateral aspect of the thigh than when it was administered in the region of the deltoid.

The height of the blood levels was much the same with both the subcutaneous and intramuscular routes. With the subcutaneous, eighty determinations were 0.1 unit per cubic centimeter or higher, and eighteen ranged from 0.04 to 0.1 unit per cubic centimeter, with the intramuscular route, sixty levels were 0.1 unit per cubic centimeter or higher, and thirteen varied from 0.04 to 0.1 unit per cubic centimeter.

It is apparent, then, that in most instances effective therapeutic concentrations of penicillin are present in the blood stream for twenty-four hours or longer following single subcutaneous injections of 600,000 units of penicillin in beeswax-peanut oil. Since absorption is equally good from the subcutaneous tissue of the thigh and the arm, many sites of injection are available in patients requiring prolonged treatment. From the height of the levels, it seems probable that most patients with infections can be treated successfully with a single daily injection. The fact that larger amounts of penicillin are required than with penicillin in saline is of little importance in view of the abundant supplies of penicillin now available and is more than compensated for by the convenience of being able to administer an entire day's treatment with a single subcutaneous injection.

SUMMARY

Effective therapeutic concentrations of penicillin are maintained in the blood stream for twenty-four hours or longer following single subcutaneous injections of 600,000 units of penicillin in beeswax-peanut oil. Results following intramuscular injections are somewhat less satisfactory than with the subcutaneous route.

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THE CONCENTRATION OF PENICILLIN IN THE SPINAL FLUID FOLLOWING INTRAMUSCULAR ADMINISTRATION IN NEUROSYPHILIS

A NEGATIVE REPORT

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THE excretion and persistence of penicillin in the spinal fluid under various conditions has been a question of considerable interest. In the early investigation of the absorption, excretion, and distribution of penicillin, Rammelkamp and Keefer¹ found none in the spinal fluid of two normal adults after intravenous injections of 10,000 units; one tested up to forty-five minutes after the injection, and the other up to 195 minutes. Herrell² administered as much as 30,000 units of penicillin intravenously in a period of fifteen minutes to a patient who did not have any demonstrable lesions of the central nervous system and could not detect any penicillin in the spinal fluid removed thirty and sixty minutes after the injection.

Penicillin injected intrathecally is slowly absorbed from the subarachnoid space and can be detected in the spinal fluid thirty-one hours later. More rapid absorption occurs from the spinal fluid of patients with meningitis, but the agent can still be found in significant amounts twenty-four hours after intraspinal administration. Because of the evidence that penicillin did not readily penetrate the barrier from blood to spinal fluid, Rosenberg and Arling,³ in a large series of patients with meningitis, utilized a combination of intrathecal with intravenous and/or intramuscular penicillin therapy. However, they were among the first to suggest that, when penicillin would ultimately be prepared in a more concentrated and a more highly purified form, free from pyrogens, it might be excreted into the subarachnoid space in sufficient amounts following intramuscular or intravenous administration to justify the abandonment of intrathecal therapy. Dawson and Hobby⁴ mentioned that, although in normal persons little or no penicillin is excreted in the cerebrospinal fluid, detectable amounts may be found in the presence of active inflammation. No data supporting their statement was offered at the time.

In August, 1944, Rosenberg and Sylvester⁵ first reported detectable concentrations of penicillin in the spinal fluid after intravenous and intramuscular administration in eight patients with meningitis. In these patients, penicillin was administered in doses of from 20,000 to 40,000 Oxford units, and from 60 to 140 minutes later penicillin was found in the spinal fluid in concentrations varying

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from 0.03 to 0.35 unit per cubic centimeter. This report was the first to demonstrate that penicillin administered other than intrathecally, in adequate dosages, may be effective in the treatment of meningitis without necessitating the supplementary route of administration. Confirmatory results were published by Cooke and Goldring,⁶ whose observations were conducted on infants from 4 to 34 months of age receiving relatively much larger doses of penicillin (from 500 to 5,000 units per kilogram of body weight) than usually given to adults. Nine patients were included in their study, five of whom had acute meningitis; three, acute nephritis; and one, acute pericarditis. All were given penicillin intramuscularly, except one patient who received an intraperitoneal injection. In four of the nine patients, from 0.1 to 0.4 unit of penicillin per cubic centimeter was found in the spinal fluid from fifteen minutes to one hour after the intramuscular injection. All of these patients had acute meningitis. In the one patient given penicillin intraperitoneally, 0.2 unit per cubic centimeter was found four hours after the injection. These observations illustrated that, when relatively large doses of penicillin are given intramuscularly, the drug may be found in the spinal fluid within fifteen minutes to one hour but tends to disappear after one and one-half hours.

In spite of the fact that penicillin has been actively used in the therapy of neurosyphilis during the past few years, there have been no published reports of studies on its excretion in the spinal fluid in patients so treated. If inflammatory changes, such as those existing in cases of meningitis, are responsible for the increased penetration of penicillin into the spinal fluid, it would not seem too unlikely that in neurosyphilis, with its various combinations of meningeal, vascular, and parenchymatous central nervous system changes, similarly increased concentrations of penicillin might be obtained and the necessity for cumbersome intrathecal administration avoided. There is, of course, also the possibility that since neurosyphilis is believed by many to be primarily a vascular disease, the penetration of penicillin through the blood-spinal fluid barrier may not actually be necessary.

In an effort to determine whether penicillin significantly penetrates into the spinal fluid in neurosyphilitic patients with different spinal fluid syndromes, this study was undertaken.

MATERIAL AND METHOD

The routine course of therapy at this neurosyphilis center from May to November, 1945, consisted of the simultaneous administration of therapeutic malaria and 3,600,000 units of penicillin over a period of fifteen days. Penicillin, in doses of 30,000 units, was injected intramuscularly every three hours, day and night, until a total of 120 injections had been given. This therapy was not begun until the patient developed his first elevation of temperature above 102° F. from the malaria infection. Twelve patients were included in the study, and a total of twenty-five observations was made. Since the degree of involvement of the central nervous system in neurosyphilis conceivably has an effect on the concentration of penicillin in the spinal fluid, cases of both asymptomatic and symptomatic neurosyphilis (meningovascular, tabes dorsalis, and dementia

paralytica) were included. Similarly, since the active meningeal inflammation, as in the previously reported cases of bacterial meningitis, probably greatly influences the distribution of the drug, both active (elevated cell counts) and inactive spinal fluid syndromes were chosen for the investigation. Specimens of blood, spinal fluid, and urine were obtained for penicillin assay at different intervals from 10 to 150 minutes following the intramuscular injection of 30,000 units. Most of the patients had previously received between 1,800,000 and 3,510,000 units before these observations were recorded.

Method of Penicillin Assay.—

Materials Used: Sterile saline; sterile pipettes (0.1 c.c. and 1 c.c.); chemically clean Wassermann and Loeffler's tubes; stock solution of penicillin (10,000 units per cubic centimeter); Wassermann racks; sterile brain-heart infusion broth (4 c.c. per tube); a known sensitive organism (*Staphylococcus aureus hemolyticus*)—a broth suspension of approximately 600,000 per cubic centimeter was used as the inoculum.

Determination of the Organism Sensitivity: Thirteen tubes of sterile brain-heart infusion broth were placed in a rack. Penicillin was added to each tube in the amounts shown in Table I.

TABLE I

TUBE	PENICILLIN (UNITS)
1	0.15
2	0.20
3	0.25
4	0.30
5	0.40
6	0.50
7	0.60
8	0.80
9	1.0
10	1.5
11	2.5
12	5.0
13	None Control

Each tube was inoculated with 0.1 c.c. of a broth suspension of *Staph. aureus* and incubated for twenty-four hours at 37° C. The organism was found to be sensitive to 0.25 unit of penicillin.

Procedure: Ten tubes of sterile brain-heart infusion broth were placed in a rack. The body fluid was added to the tubes in the amounts shown in Table II.

TABLE II

TUBE	BODY FLUID (C.C.)
1	0.1
2	0.2
3	0.3
4	0.5
5	0.8
6	1.0
7	1.3
8	1.5
9	2.0
10	None Control

Each tube was inoculated with 0.1 c.c. of broth suspension of *Staph. aureus*. The racks were incubated for twenty-four hours at 37° C. The racks were removed at the end of incubation and read. The tube which contained the smallest amount of body fluid and contained no growth was taken to indicate the amount of penicillin present. (Example: No growth in Tube 4; therefore, 0.5 c.c. of body fluid equals 0.25 unit of penicillin; 1 c.c. equals 0.5 unit.)

TABLE III. PENICILLIN CONCENTRATION IN BODY FLUIDS FOLLOWING INTRAMUSCULAR ADMINISTRATION IN NEUROSYPHILIS

CASE	CLINICAL DIAGNOSIS	CEREBROSPINAL FLUID			NUMBER OF MINUTES AFTER 30,000 UNITS I.M.	BLOOD SERUM (CONC.*)	SPINAL FLUID (CONC.)	URINE (CONC.)
		CELL COUNT (PER C.MM.)	TOTAL PROTEIN (MG. %)	WASS.				
1	Asymptomatic	51	28.0	+0.25	60	—	-.25	—
2	Tabes dorsalis	0	40.0	+0.5	90	—	-.25	—
3	Asymptomatic	10	38.5	+0.25	30	-.25	-.25	—
4	Asymptomatic	1	23.0	+0.5	150	-.125	-.125	—
5	Asymptomatic	3	45.0	+0.5	120	-.25	-.125	—
6	Asymptomatic	3	19.0	+0.5	60	—	-.125	—
7	Meningovascular	1	45.0	+0.1	30	—	-.125	—
8	Dementia paralytica	46	64.0	+0.1	100	—	-.125	—
8	Dementia paralytica	46	64.0	+0.1	20	+7	-.125	—
8	Dementia paralytica	46	64.0	+0.1	45	+1.0	-.125	—
8	Dementia paralytica	46	64.0	+0.1	70	—	—	+2.5
9	Meningovascular	9	45.0	+0.5	60	—	-.125	—
9	Meningovascular	9	45.0	+0.5	10	+6	-.125	—
9	Meningovascular	9	45.0	+0.5	40	+1.3	-.125	—
9	Meningovascular	9	45.0	+0.5	65	—	—	+2.5
10	Asymptomatic	25	92.0	+0.1	90	—	-.125	—
10	Asymptomatic	25	92.0	+0.1	50	+7	-.125	—
10	Asymptomatic	25	92.0	+0.1	70	+1.3	-.125	—
10	Asymptomatic	25	92.0	+0.1	85	—	—	+2.5
11	Asymptomatic	1	23.0	+0.25	40	+7	-.125	—
11	Asymptomatic	1	23.0	+0.25	65	+1.0	-.125	—
11	Asymptomatic	1	23.0	+0.25	80	—	—	+2.5
12	Asymptomatic	64	38.0	+0.1	30	+7	-.125	—
12	Asymptomatic	64	38.0	+0.1	55	+1.0	-.125	—
12	Asymptomatic	64	38.0	+0.1	75	—	—	+2.5

*Conc., Concentration of penicillin in body fluid. For example, +7 indicates presence of penicillin in .7 unit per cubic centimeter; -.125 indicates no penicillin within the limits of sensitivity of the tests employed (less than .125 unit per cubic centimeter).

This technique would detect a penicillin concentration as low as 0.125 unit per cubic centimeter. In Table III, a plus sign before the recorded concentration indicates the presence of the drug in that amount. A minus sign before the observation indicates that no penicillin was detected in the concentration tested. Body fluids from an untreated patient were used as negative controls. Body fluid treated with the stock penicillin was used as positive control. This procedure was applicable to urine, blood, and spinal fluid. The assays were done immediately after the collection of specimens.

RESULTS AND DISCUSSION

Of the twelve patients included in this investigation, eight had asymptomatic neurosyphilis and showed various degrees of inflammatory spinal fluid activity represented by elevated cell counts from 1 to 64 cells per cubic millimeter.

Two were patients with meningovaseular neurosyphilis, manifested by several mild neurologic changes, including abnormal pupillary reactions, hyperactive deep tendon reflexes, and bizarre motor or sensory changes. One patient with tabes dorsalis and one with dementia paralytica were also studied. Unfortunately, no patients with acute syphilitic meningitis, similar to the acute syndromes previously reported, came under treatment during the course of this analysis. At any rate, the different degrees of central nervous system involvement studied represented a series of cases more typically encountered in the penicillin treatment of neurosyphilis.

In Table III are listed the results of penicillin assay on spinal fluid, blood serum, and urine at intervals varying from 10 to 150 minutes following the intramuscular injection of 30,000 units. Several observations on the same patient indicate a serial study, in which the lumbar puncture needle was left in place, and spinal fluid specimens removed, at the intervals noted, simultaneously with the removal of blood. In several of the earlier cases studied, only single specimens were obtained. The results indicate that penicillin, within the limits of sensitivity of the assay test (0.125 unit per cubic centimeter), could not be detected in the spinal fluid in any of the patients. The reports of penicillin concentration in the spinal fluid of patients with meningitis were based on approximately the same unit sensitivity.

It has been previously emphasized that the usual methods employed in studying the absorption or excretion of penicillin are rather crude, since the selection of the procedure, in which the killing power of blood or other fluids containing penicillin is determined against the test organism, may give information of practical value in estimating the higher levels of the drug in that fluid but give no information regarding the biologic activity of material containing only traces of penicillin. Most investigators believe that this activity is very considerable and of great importance. It has, for example, been shown that penicillin induces a profound change in the morphology and growth of hemolytic streptococci in dilutions as high as one part in a billion, far beyond the zone of demonstrable bactericidal or bacteriostatic properties. It has yet to be determined whether these changes are of clinical significance.

The excellent results reported with the use of intramuscular penicillin therapy in patients with acute syphilitic meningitis⁷ warrant further study to determine whether concentration is greater in these patients or whether penicillin in therapeutically adequate amounts, but not detectable by ordinary assay methods, is excreted in the spinal fluid. Since definite improvement has been shown to occur in many other patients with neurosyphilis^{8, 9} after intramuscular penicillin, there is still the real possibility that the excretion of penicillin through the blood-spinal fluid barrier may not be necessary for its proper action in this disease. Certainly, the difficulties and occasional complications following the intrathecal administration of penicillin have resulted in increasing efforts to justify its administration by the intramuscular route in patients with neurosyphilis requiring prolonged and intensive therapy.

SUMMARY

Penicillin was administered intramuscularly in doses of 30,000 units to twelve subjects with neurosyphilis. Patients with asymptomatic neurosyphilis with varying degrees of spinal fluid cellular activity, meningovascular neurosyphilis, tabes dorsalis, and dementia paralytica were included in the study. Examination of the spinal fluid in these patients 10 to 150 minutes following injection did not reveal the presence of penicillin within the limits of the test employed (penicillin less than 0.125 unit per cubic centimeter).

Since beneficial results often follow the use of intramuscular penicillin in neurosyphilis, the drug may penetrate the spinal fluid in therapeutically effective amounts, not detectable by the methods employed, or penetration may not be necessary in a disease which is essentially vascular in nature.

Similar studies in patients with acute syphilitic meningitis may be of interest.

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PLAGUE: TREATMENT OF EXPERIMENTAL ANIMALS WITH STREPTOMYCIN, SULFADIAZINE, AND SULFAPYRAZINE

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THE difficulties which attend the prevention and the successful treatment of plague stimulate continued studies of the therapeutic value of new drugs as they are developed. In 1944¹ we reported our results of treating experimental animals with sulfadiazine after the animals had developed plague. Hornibrook² conducted preliminary tests in this laboratory with streptomycin in the treatment of white mice which were inoculated subcutaneously with *Pasteurella pestis* and concluded that it had therapeutic value under the circumstances of his experiments.

This report is a record of further investigations to evaluate streptomycin in the therapy of plague induced in mice and guinea pigs by inoculation and in guinea pigs which were infected by the bites of infectious fleas and to make comparisons of the results obtained by the use of this drug, and by sulfadiazine or sulfapyrazine.

METHODS

Mice weighing approximately 20 grams each were selected from an inbred white Swiss colony. The guinea pigs were from dealer's stocks but had been under observation long enough to determine that they were healthy; those chosen weighed about 400 grams each. Plague was induced by inoculation in both mice and guinea pigs and by natural transmission through fleas to guinea pigs. The strain of *Past. pestis* in the inoculum was one which had been isolated during the year from the sputum of a patient who had pneumonic plague and had been maintained on blood tryptose agar. The virulence of *Past. pestis* for these animals had recently been proved, and it was freshly isolated from an infected mouse immediately previous to the commencement of each series of experiments. Streptomycin was dissolved in physiologic saline solution in concentrations which permitted a variation of the dose in a constant volume of 0.2 c.c. for mice and 1.0 c.c. for guinea pigs. The drug was given subcutaneously. The sodium salts of the sulfa drugs were similarly prepared and injected subcutaneously in 10 mg. doses in mice and 100 mg. doses in guinea pigs, as the initial dose to treatment with the respective sulfa compound. The sulfa compounds were suspended in acacia with double the dose of sodium bicarbonate and administered to the mice by mouth through a syringe. The guinea pigs received the compounds and soda by mouth in capsules or in a suspension in acacia.

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‡Furnished through Dr. D. F. Robertson, Associate Medical Director of Merck & Company, Inc., New York, N. Y., who also furnished records of experimental determinations of its toxicity to small animals and information concerning the dose tolerance of the animals over stated periods.

Many of the mice recovered under the treatment adopted with each drug. It seemed evident, however, that large numbers or many lots would be required to overcome the practical difficulties inherent in the use of these animals. They exhibit no clinical changes by which the infection can be detected readily, and bleeding them from the heart often causes irreparable damage. Furthermore, it is often impracticable to repeat all the conditions prevailing in tests on each of many lots. On the other hand, the guinea pig is very susceptible to plague, and one can easily obtain good clinical evidence of infection after both inoculation and flea transmission. It seemed desirable, therefore, to attempt to confirm in guinea pigs the results obtained in the mice before making any appraisals of the therapeutic value of the drug.

TESTS ON INOCULATED GUINEA PIGS

The guinea pigs were tested in pairs. Treatment was given to the sicker of the two from forty-eight to seventy-two hours after inoculation, and the other animal served as a control. A rise in temperature above 39.3° C. and/or the development of a bubo in a lymph node adjacent to the site of the intracutaneous inoculation on the abdomen was regarded as evidence of infection. Animals were discharged after they had survived for twenty-one days after inoculation or for ten days after apparent recovery.

Five guinea pigs were each given from 20,000 to 26,000 units of streptomycin in 2,000 unit doses twice daily for six days and seemed to be recovering. Their temperatures had fallen to within a normal range and the buboes had decreased in size. Treatment was stopped. Forty-eight hours later all had either a rise in temperature or an increase in the size of the bubo. Two had developed a bacteremia. Treatment was repeated and during the following five to six days each received from 22,000 to 32,000 units and again appeared to have recovered. Ten days later, at necropsy, one animal had a soft purulent bubo from which *Past. pestis* was isolated. The others had some induration in and about the lymph nodes, but microorganisms were not obtained by cultural methods. The five controls died with plague within twelve days after inoculation.

Tests were made hourly on the blood of four guinea pigs which were given a single dose of 1,000 or 2,000 units of streptomycin in saline, or peanut oil, to determine the blood level of the drug. The method of Randall and associates³ for the determination of amounts of penicillin in the blood was used. No streptomycin was found after three hours.

In a second experiment, nineteen pairs of guinea pigs were inoculated intracutaneously, and treatment with streptomycin was started from forty-eight to seventy-two hours later on one-half of them after fever and buboes had developed and a blood culture had been made on each. An initial dose of 3,000 units was followed by 2,000 unit doses repeated twice at intervals of two hours and thereafter at intervals of four, six, and eight hours through ninety-six hours. Treatment was then stopped, and three animals had a rise in temperature. Therefore all were given 2,000 units three times daily until apparent recovery from three to eight days later. The total amounts given individual animals varied from 39,000 to 72,000 units. All survived twenty-

five days from date of inoculation, although four had a bacteremia before treatment. Eleven showed no evidence of plague at necropsy. Seven had purulent buboes and one which had had a bacteremia had both a bubo and some lung lesions. *Past. pestis* was isolated from all the buboes and the lung lesion. Eighteen controls died with plague within nineteen days after inoculation. The survivor had no evidence of plague at necropsy on the twenty-fifth day, although it had had both fever and a bubo. The range of the amounts of the drug used primarily and with recurrences, and the results obtained in the animals, are charted in Table II. Examples of the different types of the temperature curves are shown in Fig. 1.

Five normal guinea pigs were each given streptomycin in amounts and dosage similar to those used in the test animals and appeared normal when examined at necropsy on the twenty-fifth day after their first dose.

Guinea pigs apparently tolerated the therapy well and recovered from plague under it even though they had developed a bacteremia before it was started. However, recovery did not occur as promptly as it did with the use of sulfadiazine in the tests previously reported.¹

TESTS ON GUINEA PIGS INFECTED BY FLEA TRANSMISSION

Plague is primarily a disease of rodents, and its mode of occurrence in Nature can be reproduced in the laboratory under controlled conditions by the bites of infected fleas. Thus, tests can be made of a therapy in the disease acquired in a natural manner and should afford a better evaluation of it and of the possibilities of its application in cases in human beings.

Four experiments were made with 106 guinea pigs which were infected with plague by the bites of fleas (*Xenopsylla cheopis*) that had been proved to be infectious, after feeding on a mouse artificially infected, with the strain of *Past. pestis* previously used. The general conditions, administration of the drugs, and division of the pairs for therapy and control were carried out as closely as possible to those previously used. Treatment was started when the clinical evidence of infection seemed assured, usually from three to five days after the flea bite. A blood culture was made of each animal at this time.

Fifteen animals were treated with streptomycin in 2,000 unit doses given three or four times daily, in accordance with their clinical responses, until apparent recovery. The first period of treatment extended for from eight to fourteen days, and the different individuals received from 46,000 to 92,000 units. Five had recurrences and were again treated for from two to eleven days, during which time they received from 4,000 to 57,000 units. Two had a second recurrence and received from 27,000 to 46,000 units over a period of from five to eight days. Fourteen survived for ten days after their clinical recovery and were then examined at necropsy. Two had soft buboes containing viable *Past. pestis*. One died of streptococcus pneumonia after having apparently recovered from plague during the first series of treatments. Six of the fourteen survivors had a bacteremia when treatment was started. There were twelve controls and all died with plague within ten days of the development of clinical findings.

TABLE II. SHOWING RESULTS OF TREATMENT OF GUINEA PIGS WITH STREPTOMYCIN AFTER PLAGUE HAD BEEN INDUCED BY INTRACUTANEOUS INOCULATION OF PAST. PESTIS* (AMOUNTS OF DRUG RECORDED IN 1000 UNITS)

TOTAL	NUMBER OF GUINEA PIGS			REMARKS	AMOUNT OF DRUG IN FIRST TREATMENT	NUMBER OF GUINEA PIGS WITH RECURRENCE	AMOUNT OF DRUG IN SECOND TREATMENT	TOTAL AMOUNT OF DRUG
	WITH BAC- TEREMIA*	SURVIVED	DIED	WITH PLAQUE AT NECROPSY				
5 treated with streptomycin	0	5	0	1	Purulent bubo	5	22.32	44.57
5 controls	0	0	5	5				
19 treated with streptomycin	4	19	0	8	Purulent bubo in seven; purulent bubo and lung lesions in one	12	14.22	39.72
19 controls	1	1	18	18				

*Bacteremia determined by culture of heart blood before treatment.

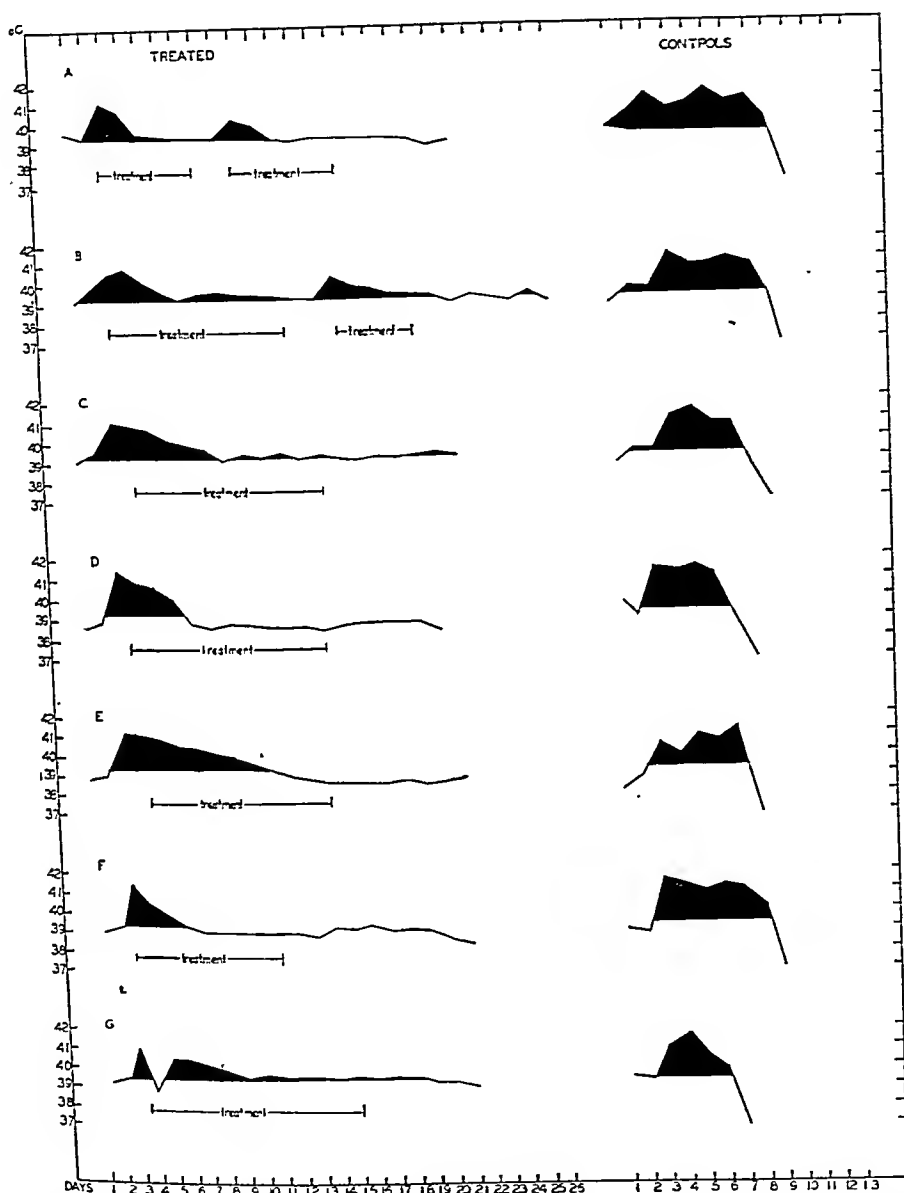


Fig. 1.—Types of fever charts of guinea pigs infected with plague and treated with streptomycin; sulfadiazine; sulfapyrazine; streptomycin and sulfadiazine; and of untreated controls. (Fever charts were made of every guinea pig.)

Treated: A, B, C, Curves of 87 per cent streptomycin; B, curves of 60 per cent sulfapyrazine; D, E, curves of 93 per cent sulfadiazine; E, curves of 30 per cent sulfapyrazine; F, curves of 50 per cent sulfadiazine and sulfapyrazine; G, curves of five very sick with bacteremia, three treated with streptomycin, one with sulfapyrazine, one with streptomycin and sulfadiazine.

Controls: A to G, Types of various curves which occurred.

Fifteen were given sulfadiazine in 100 mg. doses once or twice daily for from six to fifteen days, after which no further treatment was indicated. The total amounts given individuals varied from 500 to 3,700 mg. All received an initial dose of 100 mg. of the sodium salt. Fourteen survived for from twenty to thirty-one days and showed no evidence of plague at necropsy. One died of plague on the second day of its treatment. This animal and six others had a bacteremia before treatment was commenced. There were fourteen controls and thirteen died within fifteen days after infection. The survivor did not have plague at necropsy on the twenty-fourth day, although it had previously had a large soft bubo.

Sixteen were treated with sulfapyrazine, and individual animals received from 500 to 8,000 mg. in 100 mg. doses given once or twice daily for from four to eighteen days. All were given an initial dose of the sodium salt, and seven received from 100 to 200 mg. additionally. Ten survived for from twenty to thirty-one days; four of these had buboes containing viable *Past. pestis* at necropsy. Five died of plague between the fourth and tenth day and one on the eighteenth day with extensive lung lesions, after having apparently recovered. Six of the lot had a bacteremia before they were treated. Fifteen of the sixteen controls died of plague between the third and sixteenth day after infection. One survived twenty-five days and did not have plague at necropsy although it had clinical findings previously.

Ten were treated with both streptomycin and sulfadiazine. The dosage was the same as that used with the single respective drug. Individual animals received amounts of from 18,000 to 26,000 units of streptomycin and from 1,000 to 2,000 mg. of sulfadiazine during periods of from five to eleven days. Two animals had a clinical recurrence and were given, respectively, 900 and 2,400 mg. of sulfadiazine in the course of from two to seven days. All the animals survived for from twenty to twenty-nine days, and eight did not have plague when examined at necropsy. Two had soft buboes containing viable *Past. pestis*. Three had a bacteremia before treatment. There were eight controls and seven died with plague from four to twelve days after infection. One survived twenty days and at necropsy had a soft bubo containing viable *Past. pestis*. The number of animals treated, the amounts of the drugs administered, the recurrences, and the survivals or deaths of animals are charted in Table III. Examples of the temperature curves are shown in Fig. 1.

COMMENT

These experiments indicate that streptomycin has definite value in the treatment of white mice and guinea pigs after they have been infected with plague. It seems, however, to have less value in the treatment of mice which have developed a bacteremia than in the treatment of guinea pigs. The amounts and dosage of the drug seemed to be well tolerated by the animals for the periods of treatment. Among both the inoculated and the naturally infected guinea pigs there were a number of clinical recurrences after the animal had apparently recovered. It is possible that the recurrences may be corrected by the adoption of different methods of administration or of amounts

TABLE III. SHOWING RESULTS OF TREATMENT OF GUINEA PIGS WITH (A) STREPTOMYCIN, (B) SULFADIAZINE, (C) SULFAPYRAZINE, AND (D) STREPTOMYCIN PLUS SULFADIAZINE AFTER PLAGUE HAD BEEN INDUCED BY BITES OF INFECTIOUS FLEAS (AMOUNTS OF STREPTOMYCIN RECORDED IN 1,000 UNITS; SULFA DRUGS, IN 1,000 MG.)

TOTAL	NUMBER OF GUINEA PIGS				REMARKS	AMOUNT OF DRUG IN FIRST TREATMENT	GUINEA PIGS WITH A RECURRENT RENOE	AMOUNT OF DRUG IN SECOND TREATMENT	GUINEA PIGS WITH A RECURRENT RENOE	AMOUNT OF DRUG IN THIRD TREATMENT	TOTAL AMOUNT OF DRUG
	WITH INACT. EMIA *	SURVIVED	DIED	WITH PLAGUE AT NECROPSY							
15 treated with streptomycin	6	18-13 days 14	26th day 1	2	One pig died on twenty-sixth day with streptococic pneumonia	46-92	5	4-57	2	27-16	48-125
12 controls	6	0	12	12							
15 treated with sulfadiazine	7	20-31 days 14	5th day 1	1	One died on second day of treatment, fifth day after inoculation	0.5-3.7	0				0.5-3.7
14 controls	5	24 days 1	13	13	One developed bubo and fever but recovered						
16 treated with sulfapyrazine	6	20-31 days 10	6	10	Four had purulent buboes only but with viable P. pestis	0.5-2.85	6	0.9-2.5	0		0.5-8.0
16 controls	7	25 days 1	15	15	One developed bubo and fever but recovered						
10 treated with (a) streptomycin and (b) sulfadiazine	3	20-29 days 10	0	2	Two had soft purulent buboes only but with viable P. pestis	(a) 18-26 (b) 1-2	2	(b) 0.9-2.4	0		(a) 18-26 (b) 1.0-4.4
8 controls	1	20 days 1	7	8	One had soft purulent bubo only but with viable P. pestis						

* Inactoremia determined by culture of heart blood before treatment.

and timing of the dosage, since it appears that the drug remains in the blood stream for a very short period. In several instances, a soft purulent bubo remained after the apparent recovery of a guinea pig treated by streptomycin or by another of the drugs. In the light of experience with plague in rodents, it may be assumed that most of such animals had recovered and that the bubo would have healed and the microorganisms would have been eliminated without further therapy.

The results of the treatment of guinea pigs with sulfadiazine are confirmatory of those previously reported by us. Although sulfadiazine appeared to bring about recovery more promptly than did streptomycin, it does not seem that comparison of the two is justified because of the reasons previously expressed.

The use of a combination of streptomycin and sulfadiazine in treatment does not seem to add to the effectiveness of either. Sulfapyrazine offers no advantages over sulfadiazine.

The difficulties of appraising the progress of plague in white mice, and the rapidity with which they develop bacteremia and die, necessitates the use of many lots of animals with the probabilities of the introduction of different conditions while making an adequate test of a therapy. For these reasons it is believed that remedies to be used in plague should be tested on other animals.

SUMMARY

1. Three hundred and thirty-four white mice were inoculated with plague, and one-half were treated in groups with different amounts and varying dosage of either streptomycin, sulfadiazine, or sulfapyrazine. The other one-half were used as controls. Under the treatment schedules finally adopted, from sixteen to eighteen survived among each lot of from eighteen to twenty, while all the corresponding controls died with plague.

2. Forty-eight guinea pigs were likewise inoculated, and one-half were treated with streptomycin in two groups after clinical evidence of infection had developed. All survived, but eight had residual buboes at necropsy. Twenty-three controls died with plague.

3. One hundred six guinea pigs were infected through flea transmission. After the disease was evident clinically, fifteen were treated with streptomycin and fourteen survived; fifteen received sulfadiazine and fourteen survived; ten received both these drugs and all survived; sixteen received sulfapyrazine and ten survived. Six among all the survivors had residual buboes at necropsy. Three survived among fifty controls divided among the four lots treated.

4. Clinical recurrences appeared frequently under the treatment schedules used with streptomycin and sulfapyrazine but not with that of sulfadiazine.

We wish to thank Cleon W. Abbott, Medical Technician, for the technical assistance given.

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THE EFFECT OF GOLD CHLORIDE ON PLASMA ASCORBIC ACID IN THE RAT

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THE complications which so frequently attend the clinical use of gold compounds suggest the value of investigating the effect of such substances on experimental animals. In the present series of experiments, gold was employed in one of its simplest forms, yellow gold chloride. It was administered by intraperitoneal injection of 0.5 per cent aqueous solution. The rats used were of the Long-Evans strain and were fed a modified Steenbock diet.

TOLERANCE TO GOLD CHLORIDE

To determine the tolerance of the rat to yellow gold chloride, graded single doses were given to a series of rats and the survival time was noted. The results are recorded in Table I. To facilitate comparison with other gold salts, the dosage is given in terms of milligrams of gold per kilogram of body weight of the animal rather than in milligrams of gold chloride.

Single doses smaller than those listed in Table I were not lethal. With a dosage of 20 mg. of gold per kilogram, a rat weighing 215 grams tolerated daily injections for five days, dying on the sixth day. These results may be compared with those of Cortell and Richards,¹ who employed intramuscular injection in rats. They found a minimum lethal dose of approximately 13 mg. of gold per kilogram for gold sodium thiosulfate, while 37 mg. of gold per kilogram were tolerated if administered in the form of gold sodium thiomalate.

On the basis of these results, it was concluded that a dosage of 15 mg. of gold per kilogram would be suitable for the repeated intraperitoneal administration of gold chloride to rats. In the course of the chronic experiments, it was found that an increase in tolerance had developed. This is illustrated by a rat which received nine injections of 15 mg. of gold per kilogram, five of 20 mg. per kilogram, and, finally, five of 25 mg. per kilogram before succumbing. Of two other rats, one survived eleven and the other thirteen doses of 20 mg. of gold per kilogram, after having previously received fourteen and nine doses, respectively, of 15 mg. per kilogram. This increase in tolerance to gold chloride is similar to the development of tolerance to gold sodium thiosulfate reported by Cortell and Richards.¹

The gold-treated animals lost weight during the first three days of treatment, the average loss being 10 grams per animal. Following this loss, however, they again started gaining weight, the average increase being 2.0 grams per day for the rats receiving 15 mg. of gold per kilogram, compared with 2.4 grams per day for the controls. Increasing the dosage of gold decreased the

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TABLE I. SURVIVAL TIME AFTER SINGLE INTRAPERITONEAL DOSES OF GOLD CHLORIDE

WEIGHT OF RAT (GM.)	DOSE (MG. GOLD KG.)	SURVIVAL TIME (HR.)
125	50	2
175	40	5
202	30	7
186	28	8
178	25	15
190	25	40
187	23	51

growth rate. The figures given do not include the terminal period of existence, during which abdominal distention following renal failure frequently occurs, together with a marked loss in weight.

PLASMA ASCORBIC ACID OF GOLD-TREATED RATS

The effect of gold chloride administration on the level of ascorbic acid in the plasma was determined in two series of rats. In the first experiment, the ascorbic acid was determined from samples of blood drawn two days after the last injection of gold chloride. The determinations of ascorbic acid were made by the method of Farmer and Abt²; the data are given in Table II.

Although the results of this experiment definitely indicate a decrease in the ascorbic acid content of the plasma of the rats receiving gold chloride, it was thought advisable to repeat the experiment, obtaining initial ascorbic acid values for some of the experimental animals. The major portion of this experiment was terminated at the end of two weeks, but two of the experimental animals were kept until the end of five weeks. During the first two weeks, each rat received nine intraperitoneal injections of 15 mg. of gold per kilogram. The two animals kept under treatment during the following three weeks received, in addition, five injections of 15 mg. per kilogram and eight of 20 mg. per kilogram, the total administered during the five weeks being 370 mg. of gold per kilogram. The decrease in plasma ascorbic acid in the gold-treated rats is shown by Table III. Death intervened due to the effect of the gold on the kidneys before definite scorbutic symptoms were observable.

Similar results were obtained in preliminary experiments on guinea pigs. In two guinea pigs receiving seven injections of 10 mg. of gold per kilogram during a ten-day interval, the plasma ascorbic acid dropped from 0.6 and 0.5

TABLE II. PLASMA ASCORBIC ACID OF GOLD-TREATED RATS; FIRST EXPERIMENT: GOLD CHLORIDE ADMINISTERED DAILY; PLASMA ASCORBIC ACID DETERMINED TWO DAYS AFTER LAST DOSE OF GOLD CHLORIDE

TREATMENT	TOTAL GOLD (MG. KG.)	FINAL PLASMA ASCORBIC ACID (MG. %)
Average of 11 controls (range of ascorbic acid, 0.78 -1.32 mg. %)	0	1.04
Seventeen doses of 2.0 mg./kg.	34	0.82
Five doses of 0.6 mg./kg. and eleven doses of 4.0 mg./kg.	47	0.82
Six doses of 1.3 mg./kg. and eleven doses of 9.0 mg./kg.	107	0.66
Seven doses of 7.5 mg./kg.	53	0.49
Seven doses of 10.0 mg./kg.	70	0.49

TABLE III. PLASMA ASCORBIC ACID OF GOLD-TREATED RATS; SECOND EXPERIMENT

	PLASMA ASCORBIC ACID (MG. %)		
	INITIAL	2 WK.	5 WK.
Controls	1.32	0.67	0.86
	0.78	0.57	0.75
Gold-treated rats	1.16	0.26	0.13
	--	0.38	0.28
	1.09	0.33	
	1.01	0.46	
	--	0.41	
	1.01	0.27	
	--	0.39	

During the first two weeks nine doses of 15 mg. gold per kilogram were given; the two animals which were kept for five weeks received five additional doses of 15 mg. gold per kilogram and eight of 20 mg. gold per kilogram.

mg. per cent to 0.0 mg. per cent. The control showed 0.8 mg. per cent of plasma ascorbic acid at the beginning of the experiment and 0.5 mg. per cent after ten days. Data from such a small number of animals are significant only in that they indicate that the reaction of the guinea pig to gold chloride is similar to that of the rat.

COMMENT

The depletion of the plasma ascorbic acid of the rat by the administration of gold chloride is especially interesting since the rat is able to *synthesize* ascorbic acid. If the rat alone suffered this depletion upon gold treatment, there would be a possibility that the gold affected the process of synthesis. The experiments on guinea pigs, however, indicate that in these animals also the administration of gold chloride is followed by decrease in plasma ascorbic acid. A decrease in vitamin C in certain tissues, notably adrenal, lung, and duodenum, has been reported by Sande³ after administration of Sanocrysin.

The mechanism by means of which the depletion takes place is unknown. It is possible that the gold chloride may oxidize the ascorbic acid directly, a reaction which proceeds with facility in the test tube. If this reaction takes place in the body, it might be expected to lead to an accumulation of metallic gold in the cells which contain active ascorbic acid. Such deposits might well be dense enough, on occasion, to be visible under the microscope. Histologic studies of the distribution of gold in the tissues, to be reported in detail later, do not reveal a parallelism between the distribution of gold and of ascorbic acid. The depletion of ascorbic acid may be part of a general toxic reaction rather than a specific effect of gold chloride. In either case, the recognition of its occurrence after gold chloride administration is of value.

SUMMARY

The plasma ascorbic acid of the rat is markedly decreased by the intraperitoneal administration of gold chloride. Repeated injections of gold result in the development of increased tolerance and a diminished growth rate.

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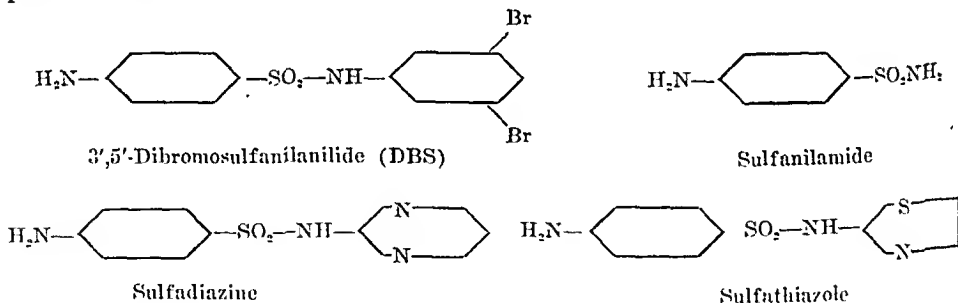
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FURTHER STUDIES ON THE ANTIBACTERIAL ACTION OF 3', 5'-DIBROMOSULFANILANILIDE

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A PREVIOUS communication from these laboratories presented the antibacterial effects of a group of benzenesulfonic acid derivatives.¹ In general, the most active members of this group were 3',5'-dibromosulfanililide and the corresponding dichloro derivative. Not only were these compounds found to be highly active against such pathogens as types I, II, and III pneumococci, *Streptococcus pyogenes* (C203), *Staphylococcus aureus*, Groups I and IIa meningococci, *Clostridium welchii*, *Clostridium tetani*, *Shigella paradysenteriae* (Hiss), and *Vibrio cholerae*, but they proved to be as effective against a sulfonamide-resistant strain of the gonococcus as against several sulfonamide-susceptible strains. In addition to the above attributes, the compounds were found to be unaffected by the presence of p-aminobenzoic acid when tested against *Str. pyogenes*.

Inasmuch as these compounds exhibited several properties not associated with the common sulfonamides (sulfanilamide, sulfadiazine, sulfathiazole, etc.), an extensive pharmacologic study was made of 3',5'-dibromosulfanililide (hereinafter referred to as DBS). The structural relationship of DBS to sulfanilamide and the two substituted heterocyclic sulfonamides used in the present study appear as follows:



The physical properties, methods of chemical determination, acute and chronic toxicities, absorption, metabolism, and excretion of DBS were investigated, and the results will be reported by McChesney, Goetchius, and Lawrence.² It was found that the toxicity of DBS was of the same order as that of sulfathiazole, which is slightly more toxic than sulfanilamide. Only minute amounts were found to be excreted in the free form in the urine of dogs and rats. In rabbits, however, the larger proportion of the dose was found in the free form in the urine. In man, the drug was excreted in a con-

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jugated form which lost some of the bactericidal potency of the original compound. Rapid and satisfactory absorption occurred in rats, inasmuch as the drug was readily demonstrated in the blood following oral administration. In man, only small amounts of free DBS appeared early in the blood, but rather large amounts of the conjugated form (from 4 to 5 mg. per cent) appeared several hours later.

These encouraging pharmacologic findings prompted a study of the antibacterial action of DBS upon several other pathogenic microorganisms. The results of this investigation are presented herewith.

ORGANISMS TESTED AND METHODS OF STUDY

The general method of study consisted of making an initial dilution of 1:1000 DBS in an appropriate broth medium and of preparing serial dilutions from this in the same broth. Due to its relative insolubility in water, DBS was first dissolved in a small amount of alcohol preparatory to the initial dilution. Control tests showed this amount of alcohol to have no inhibitory effects upon the growth of the test organisms. For purposes of comparison, sulfadiazine and sulfathiazole were dissolved directly in broth and treated in a similar manner. The tubes, containing a total of 5 c.c. (10 c.c. in the case of Brewer's fluid thioglycollate medium), were autoclaved at 10 pounds for ten minutes. After cooling, each tube of a dilution series was inoculated with a 4 mm. loopful of a twenty-four hour broth culture of one of the test organisms. The tubes were incubated at 37° C. and examined for growth after twenty-four hours. Tubes showing no visible growth or growth less than one-half of that in the drug-free control were considered to contain sufficient drug to cause bacteriostasis of the test organism. Those tubes showing no growth after seventy-two hours' incubation were subcultured by transferring 3 loopfuls to fresh medium lacking the drug. Failure of growth to appear in the subculture tube after seventy-two hours' incubation at 37° C. was taken as evidence that the organisms were destroyed in the original drug-organism-broth mixture.

TABLE I. HIGHEST DILUTION OF COMPOUND EXHIBITING ANTIBACTERIAL ACTION

ORGANISM	BROTH MEDIUM	DBS		SULFADIAZINE		SULFATHIAZOLE	
		BS	BC	BS	BC	BS	BC
<i>Gaffkya tetragena</i> (RG)	Beef extract	32,000	32,000	<1,000	<1,000	<1,000	<1,000
<i>Gaffkya tetragena</i> (MB)		64,000	32,000	2,000	<1,000	16,000	<1,000
<i>Streptococcus agalactiae</i>	Veal infusion + 0.15% dextrose	64,000	32,000	1,000	<1,000	<1,000	<1,000
<i>Listerella monocytogenes</i>		128,000	16,000	<1,000	<1,000	<1,000	<1,000
<i>Streptococcus viridans</i>	Veal infusion dex- trose + 0.1% horse serum	16,000	8,000	<1,000	<1,000	<1,000	<1,000
<i>Hemophilus ducreyi</i>		512,000	32,000	64,000	<1,000	256,000	<1,000
<i>Brucella abortus</i>	Tryptose-phosphate + 0.1% agar	64,000	16,000	32,000	<1,000	4,000	<1,000
<i>Brucella melitensis</i>		128,000	32,000	32,000	<1,000	8,000	<1,000
<i>Brucella suis</i>		256,000	16,000	32,000	<1,000	16,000	<1,000
<i>Actinomyces bovis</i> (anaerobic)	Brewer's fluid thio- glycollate	128,000	16,000	2,000	<1,000	64,000	<1,000

BS, Bacteriostatic.

BC, Bactericidal.

<1000, Concentrations greater than 1:1000 not tested.

The organisms tested, the medium employed in each case, and the extent of antibacterial action are presented in Table I.

RESULTS AND DISCUSSION

It may be noted from the data given in Table I that DBS exerts a far greater antibacterial action upon all the test organisms than does sulfadiazine or sulfathiazole. The latter two failed to show any bactericidal action in a 0.1 per cent concentration, whereas DBS was bactericidal for all the organisms.

The history of the two strains of *Gaffkya tetragena* is of more than passing interest. Both of these strains were recent isolations from individuals suffering from infection. Strain MB was isolated from a draining sinus of the hip, while strain RG was isolated from the blood stream of a patient with septicemia. Laboratory tests on strain RG produced some unusual results. This organism was completely unaffected by sulfadiazine, sulfathiazole, or sulfamerazine, even in a 1 per cent concentration of the drugs. Furthermore, 5,000 units per cubic centimeter of penicillin were required to inhibit its growth. This is another instance in which a sulfonamide-resistant organism (also penicillin-resistant) is susceptible to the antibacterial action of DBS.

The treatment with sulfonamides and penicillin of subacute bacterial endocarditis caused by *Streptococcus viridans* has met with varying degrees of success.^{3, 4} The sulfonamides have been employed successfully in the treatment of experimental infections caused by *Listerella monocytogenes*⁵ and by *Hemophilus ducreyi*.^{6, 7}

In the treatment of actinomycosis and brucellosis, sulfonamides and penicillin are of questionable value. While these agents did exert some action against *Actinomyces bovis* in vitro, Keeney and associates^{8, 9} caution that the action of sulfonamides on *A. bovis* is not spectacular and that expectancy of cure from their use in human actinomycosis should be regarded with conservatism.

In reviewing the English literature on the sulfonamide therapy of brucellosis, Urschel¹⁰ concluded that the sulfonamides are of doubtful value in the therapy of brucellosis, inasmuch as the percentage of successful therapy was no higher than might be expected among untreated patients with this disease.

From this brief summary of the status of the chemotherapy of infections caused by the organisms listed, it is apparent that in most cases the available sulfonamides fall far short of being satisfactory. A comparison has been made between DBS and two commonly employed sulfonamides. Whether or not sulfathiazole or sulfadiazine had been effective in any of the disease-conditions discussed, DBS has a superior in vitro activity against the organisms responsible for these infections, an observation which suggests its trial in vivo.

SUMMARY

1. The activity of 3',5'-dibromosulfanilamide has been compared to that of sulfadiazine and sulfathiazole against such organisms as *G. tetragena*, *Streptococcus agalactiae*, *Str. viridans*, *L. monocytogenes*, *H. ducreyi*, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, and *A. bovis*.

2. A greater bacteriostatic effect and a far greater bactericidal effect was exerted by 3',5'-dibromosulfanililide than by sulfadiazine or sulfathiazole against these organisms.

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THE APPLICATION OF THE "LYOPHILE TECHNIQUE" TO BIOLOGIC PRODUCTS

I. SERA, ANTISERA, AND ANTIBODY GLOBULINS

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IN 1931, this laboratory began work on the development of the "lyophile technique." Since that time, the process has been applied to a variety of biologic products. It is the purpose of this paper to summarize the results of this experience and to point out some of the practical limitations of the process. This phase of the subject has not received the same adequate consideration as the study of the fundamental principles underlying this method of drying and of designing a satisfactory apparatus to carry on the process.

A desiccation process, to be of practical value, must meet certain critical requirements. When applied to biologically active substances, the dehydrated product (1) must show no substantial loss of potency as a result of the desiccation process; (2) must show a permanency of preservation not only under favorable, but also under adverse, temperature conditions similar to those encountered in tropical climates; and (3) should show complete and reasonably quick solubility so that the average practitioner can administer it to the patient with the least effort and expenditure of time to himself and with the least annoyance and anxiety of waiting to the patient.

The biologic products which have been desiccated by the "lyophile technique" and which have been studied by us include experimental lots as well as products prepared under actual routine commercial conditions. Unless otherwise specified, the residual moisture content of the products was held to a maximum of 1.0 per cent or less. Examples are given from various classes of products studied and the degree to which they fulfill the three requirements previously mentioned is indicated. From these examples, we are able to make a few generalized statements relative to the application of the "lyophile technique" to biologic products and to point out its advantages and practical limitations.

Products which have been processed and studied may be classified conveniently as follows: (1) antisera (including normal guinea pig serum and human plasma); (2) antibody globulins; (3) viruses and virus vaccines; (4) live bacterial suspensions.

Antisera and antibody globulins only will be discussed in this communication.

SERA AND ANTISERA

Many investigators had previously reported on the keeping qualities of dry immunologically active substances, including antisera. Some failed to specify

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the temperature under which they were kept, and others merely stated that the particular substance in question retained its properties when kept under the favorable temperature condition of the icebox. The relationship of the moisture content of the product under preservation to keeping qualities rarely was considered and seldom was discussed.

In order to obtain this desirable information, we investigated the influence of three factors, time, temperature, and residual moisture content, on the potency of antitoxic and antibacterial sera. Diphtheria antitoxic serum was studied more thoroughly than some of the others because its potency can be determined with a fair degree of accuracy by means of the *in vitro* flocculation test and by the *in vivo* L⁺ test. The results obtained on this product are given in detail, presumption being that the conclusions derived from these results apply in a general way to the other antitoxic sera studied.

The serum was placed in 20 c.c. amounts into 50 c.c. containers and was frozen at -78° C., after which it was dehydrated on a manifold at the temperature of the surrounding air. The period of desiccation was varied purposely so that the end products would contain varying amounts of moisture. This was done by removing some of the containers from the manifold after twelve, sixteen, twenty, twenty-four and forty-eight hours of desiccation. Sample containers from each group were tested for moisture content, Lf (flocculation units), Kf (time of flocculation), and for antitoxic units by the standard guinea pig test. The results are shown in Table I.

TABLE I. "LYOPHILE" DIPHTHERIA ANTITOXIC SERUM (RESTORED IMMEDIATELY AFTER DESICCATION)

DESICCATION PERIOD (HR.)	GROUP	MOISTURE CONTENT (PER CENT)	Lf UNITS	Kf (MIN.)	ANTITOXIC UNITS GUINEA PIG TEST
12	1	15.6	750	35	700
16	2	2.6	750	37	600
20	3	1.9	750	31	600
24	4	1.4	750	25	600
48	5	1.0	750	26	700
Original liquid serum untreated			750	26	700

It is apparent that the Lf, Kf, and the antitoxic potency of the serum had not changed significantly as a result of the desiccation process.

A dozen containers (stoppered under vacuum) from each group were then stored at 5° C. and at 40° C. A sample container from each group was taken at the end of one, three, six, and thirteen months and the serum restored with the proper amount of distilled water and tested for potency (L⁺ units), Lf units, and Kf. In order to save space, the protocols of the one-, three-, and six-month aging tests are omitted. The results of the same serum groups tested after thirteen months are given in Table II.

The aging tests bring out certain interesting facts. After one month of storage at 40° C., the serum in Group 1 was no longer completely soluble and after three months' storage, was completely insoluble. The corresponding sample kept at 5° C. for three months dissolved completely and showed no loss in potency. This demonstrated very clearly that a high moisture content in

TABLE II. "LYOPHILE" DIPHTHERIA ANTITOXIC SERUM (STORED AT 5° C. AND AT 40° C. FOR THIRTEEN MONTHS)

GROUP	MOISTURE CONTENT (PER CENT)	5° c.			40° c.		
		L+ UNITS	Lf UNITS	Kf (MIN.)	L+ UNITS	Lf UNITS	Kf (MIN.)
1	15.6	700	750	60	Insoluble; jellied		
2	2.6	600	750	38	700+	750	> 13 Hr.
3	1.9	700	750	39	700	750	13 Hr.
4	1.4	600+	750	40	600	750	13 Hr.
5	1.0	700	750	42	600	750	13 Hr.

an apparently dry-looking serum leads to rapid deterioration after an exposure to a temperature of 40° C. for one month.

The serum in Groups 2 to 5 inclusive, containing from 2.6 to 1.0 per cent moisture, showed no significant change in potency after thirteen months' exposure to 40° C. That the serum in these groups did undergo some change, however, is revealed by the longer time it took to dissolve the dry serum completely and by the marked increase in the Kf or time of flocculation. The restored samples of serum showed a faint haze compared with those samples stored at 5° C. and similarly restored.

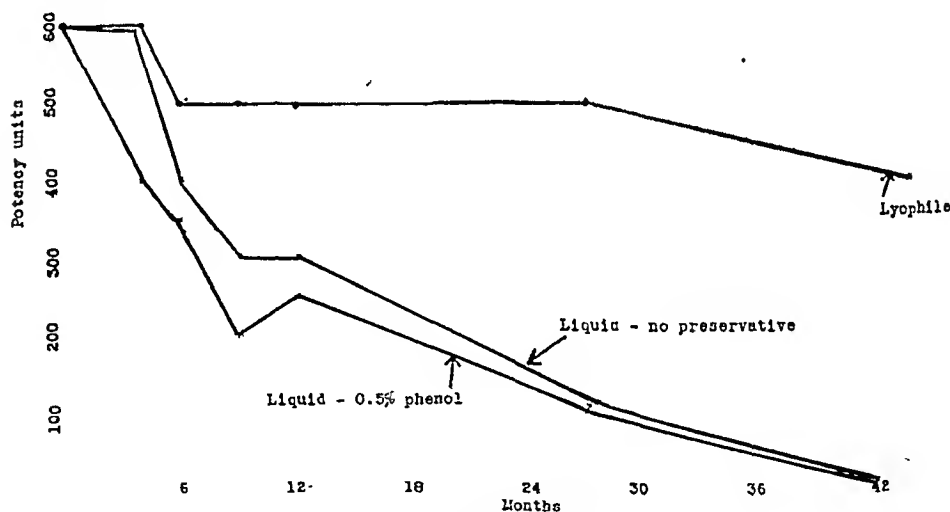


Fig. 1.—Diphtheria antitoxic serum. All samples stored at 37° C. Potency determined by animal tests.

In another experiment diphtheria antitoxic serum was divided into three parts: (1) without preservative, (2) with 0.5 per cent phenol, (3) without preservative. All three portions were filled into all-glass containers; Parts 1 and 2 were left in the liquid state and Part 3 was lyophilized and sealed under vacuum. All containers were stored at 37° C. and the potency of the serum was tested at various intervals, using the standard animal test. The results are graphically represented in Fig. 1. At this temperature, the longer preservation or retention of potency of the "lyophile" material certainly is very striking.

The influence of time, temperature, and residual moisture content on the potency of antibacterial sera and particularly antimeningococcic serum was also investigated. The "lyophile" serum and the original liquid serum were filled into suitable containers and stored at 5° C., room temperature (from 20 to 32° C.), and 37° C. Both liquid and "lyophile" sera were tested for agglutinin titer after two, three, eight, and 15 months of storage. The results obtained may be briefly summarized as follows.

After a storage period of two months at room temperature and at 37° C., the liquid sample had lost its agglutinin titer completely, whereas after storage for fifteen months at 37° C., the "lyophile" sample still retained a very substantial portion of its agglutinin titer which was about equal to that retained by the liquid sample kept at 5° C. for the same period.

The difference in physical appearance of the various samples also was impressive. All the liquid samples, regardless of the temperature of storage, showed marked physical changes manifested by a change in color and by the presence of considerable flocculent material. The "lyophile" serum samples all dissolved easily and completely and yielded a clear yellow fluid resembling in appearance the original liquid serum before it was aged.

ANTIBODY GLOBULINS

To minimize the incidence of serum reactions, it has been the practice for many years to purify and concentrate therapeutic sera. The work of McFarlane^{1, 2} and Pedersen³ points to the possibility that changes in the molecular and other properties of the serum globulins are produced by the process of purification. This and the practice of including a given dosage in a minimal volume introduce factors which influence markedly the hydrophilic properties of dry globulins.

Antitoxic globulin (diphtheria, tetanus, perfringens) preparations may be readily converted to the "lyophile" state. The resulting dry mass presents a much more compact and less friable structure than that obtained with unpurified sera. Apparently the interstices between the protein fibrous structure are not as numerous and the mass is, therefore, not as porous. On the addition of water, the diffusion of the solvent throughout the mass is relatively slow. In addition, the hydrophilic property of the globulins is less pronounced than that of whole serum. As a result, the time of dissolution or restoration of "lyophile" antitoxic globulins is greatly prolonged.

Although the dry globulins dissolve completely and the potency of the restored material is essentially that of the unprocessed material, the excessively long time required for dissolution makes their clinical use almost impractical.

The biologic activity of equine antibacterial sera (antipneumococcic, antimeningococcic, etc.) is generally contained in the less soluble euglobulin fractions. Bacterial antibody globulin preparations also "lyophilize" well and, on the addition of water, eventually dissolve completely. The restored product shows little or no loss of potency. However, whether it is due to the particularly large molecular size of the antibody protein molecule or to the relatively

lower solubility of euglobulins, or to both factors, "lyophile" bacterial antibody globulin preparations require a longer time to dissolve than do any other class of "lyophile" serum or serum derivatives. They are, therefore, the least desirable for clinical use.

Some examples of the time required to dissolve representative classes of "lyophile" serum preparations are given in Table III. Restoration time depends on several factors: nature of the dry product (serum or globulin), protein concentration of the liquid preparation, thickness of shell or surface/volume ratio of frozen mass, and uniformity of drying (absence of fused spots). It should be noted that there is often a wide variation in the restoration time between the individual containers of the same lot of a given product. This is presumably due to variations in the thickness of the shell and occasionally to the presence of a fused spot which indicates drying of the serum in that spot from the liquid state. Such fused spots take longer to dissolve.

TABLE III. RESTORATION TIMES OF VARIOUS "LYOPHILE" PREPARATIONS

DESCRIPTION OF PRODUCT	VOL. RESTORED TO C.C.	NUMBER OF CONTAINERS TESTED	RESTORATION TIME (MIN.)	REMARKS
Antimeningococcic serum, 30 c.c.	15	10	3, 6, 13, 4, 4, 4, 4, 5, 17, 4	Unconcentrated serum restored to one-half original volume
Antidysenteric serum, 50 c.c.	50	4	4, 6, 3, 4	Unconcentrated serum
Antidysenteric serum, 25 c.c.	25	2	27, 30	Serum concentrated two times prior to desiccation in market container
Diphtheria antitoxic globulin, 5 c.c.	5	4	25, 30, 40, 32	Purified and concentrated by enzymatic process; total solids, 17.7 per cent
Tetanus antitoxic globulin, 10 c.c.	10	5	25, 20, 27, 32, 30	Purified and concentrated by salting out; total solids, 17.5 per cent
Tetanus antitoxic globulin, 13.8 c.c.	8	5	5, 5, 4, 5, 6	Diluted to 6.0 per cent total solids prior to desiccation
Pneumococcic antibody globulin	10	5	All > 60	Purified and concentrated by Felton's method; total solids, 7.7 per cent

The results in Table III indicate that the most advantageous types of "lyophile" serum product in final containers for use by the clinicians appears to be whole serum. For certain antitoxic globulins, it has been found expedient to dilute the preparation to a protein content of from 5 to 6 per cent prior to desiccation. In most cases, such antitoxic globulins will dissolve within five minutes or thereabouts.

In our laboratories, a desirable limit of restoration time (average) of five minutes has been set arbitrarily for all "lyophile" products used clinically. Human plasma, guinea pig complement, and most antitoxic and antibacterial whole sera meet this standard without much difficulty.

SUMMARY

1. Unprocessed (whole) sera taken as a class are admirably adapted to the "lyophile technique." The dehydrated products show no substantial loss

of potency as a result of the desiccation process; the products are restored rapidly and completely and the preservation of potency is maintained under adverse temperature conditions.

2. The removal of water from sera by the "lyophile technique" does not completely arrest or stop all subsequent chemical changes but certainly does retard them. The dry serum forms a system which eventually proceeds to a state of equilibrium which is determined by the temperature, pressure, moisture content, and other energy factors to which the serum is subjected.

3. The maximal residual moisture content in "lyophile" sera was arbitrarily set at 1.0 per cent, a figure now accepted and adopted by the National Institute of Health. This figure seems reasonable and allows a margin of safety for "lyophile" sera kept for long periods of time at a temperature higher than 5° C.

4. The potency of "lyophile" serum is retained for a longer period as compared with the same serum stored in the liquid state. This increased stability is least at from 2° to 5° C., more marked at room temperature and very marked at 37° C.

5. The most advantageous type of "lyophile" serum produced in final containers for use by the clinicians appears to be whole serum. Antibody globulins, unless diluted to a protein content of from 5 to 6 per cent prior to desiccation, are impractical for clinical use because of the excessively long time required to dissolve them.

6. The conversion of liquid sera to the "lyophile" form involves an expense which is reflected in the cost of the product. This added cost is justified for some products but not for others. The lability of the particular product and the temperature to which it is exposed are important determining factors.

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PROGRESS

FUNDAMENTAL INFORMATION ON THE MECHANISM OF SPECIFIC TUBERCULO-IMMUNITY

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SPECIFIC tuberculo-immunity in tuberculosis has been recognized for over half a century, and yet today its significance is discounted by many, apparently because we are still unable to measure its potency in man by any known safe method. This is not surprising, however, when we realize that certain groups of tuberculologists insist that tuberculology is so primitive that the value of any chemo- or biotherapeutic agent for tuberculosis in man must be proved by experimental test in the tuberculous patient and that no amount of animal experimentation can yield satisfactory conclusions. This would be to deny the similarities and differences noted for man and the lower forms of life as of value.

In a brief history of specific tuberculo-immunity,¹ tribute was paid to the pioneers in this field: Joseph Parrot, who announced the result of investigations concerning the relationship between pathologic changes in the lungs and in the tracheobronchial lymphatic glands; Robert Koch, who demonstrated for the first time the immune phenomenon in animals; Emil von Behring, whose daring Jennerization of cattle with a "bovo-vaccine" brought condemnation because the milk for infant immunization contained viable virulent human tubercle bacilli; Paul H. Roemer, whose monumental contributions led to the view of the unity of specific immunity and tuberculin hypersensitiveness, which later guided Allen K. Krause in the same view, that tissue allergy and immunity are the attributes of animals with tubercle; Anton Ghon, whose monograph stressed the significance of the primary tubercle and was the basis of the elaborate immunobiologic conceptions of Karl Ranke; Edward L. Trudeau; Gerald B. Webb; and Calmette. Aside from the vaccination endeavor, Calmette's greatest scientific achievement was to broach the avirulence of tubercle bacilli, although he did not settle it convincingly.

TUBERCULIN

For simplicity and clarity in defining tuberculosis, a brief presentation regarding the practical significance of tuberculin is a pertinent introduction here because, in the past, tuberculin has been misleading in the clear understanding of the mechanism of tuberculosis.

Naturally tuberculin and its effects confused us a great deal, but in 1940² we noted that tuberculin was not appreciable in liquid mediums until after maximum growth of tubercle bacilli had occurred, and then it increased in the liquid to a maximum with aging of the bacillary mass in from six to ten months.

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At that time we suggested it might be a product of autolysis of the bacilli, which was definitely proved later with the preparation of autolytic tuberculin.³

In 1939⁴ we pointed out that specific immunity against virulent infections could not be produced by pure tuberculo-protein, by highly concentrated filtrates (ultradialysis), or by alum-treated filtrates, and that these materials possess no appreciable primary toxicity for normal animals. However, a primary intravenous injection of tuberculin does not sensitize to a second intracutaneous injection with a fairly large test dose (0.1 mg.) of tuberculin. A reaction to tuberculin can be obtained in a tuberculous animal with as little as 0.000,005 mg. of tuberculin. A primary intravenous injection of tuberculin (2 mg.) sensitizes to a lethal intravenous provocative dose (anaphylactically) of as little as 1 mg. of tuberculin. Production of cutaneous hypersensitiveness to tuberculin requires a small amount of avirulent bacilli, a large amount of heat-killed bacilli (100 mg.), and very small amounts of virulent bacilli (the factors of multiplication and tuberculous involvement complicate the latter picture). In 1940⁵ it was pointed out that tuberculin-sensitized animals do not develop an acute (anaphylactic) shock after the injection of washed young tubercle bacilli, while bacillary-sensitized and tuberculous (allergic) animals succumb to these injections. At the same time, it was shown that the tuberculin (anaphylactic) sensitization can be transferred passively from a high percentage of the donors, while the bacillary and tuberculous (allergic) principles are never transferable passively to normal recipients. Likewise, at that time tuberculin desensitization or immunization to the skin tuberculin reaction exerted no influence upon the specific tuberculosis immunity. The same year⁶ it was shown that the phenomenon of local skin reactivity (Shwartzman) appeared to be of no significance in tuberculosis intoxication. It was also found that animals sensitized to tuberculin do not lose this anaphylactic sensitivity upon infection with virulent tubercle bacilli and the injection of avirulent tubercle bacilli. Thus, for the first time, the two reactions, anaphylactic and allergic hypersensitiveness, were demonstrated to coexist in the same animal. In 1941⁷ it was noted that "although the specific toxicity in tuberculosis cannot be described definitely as yet, for lack of information regarding the part played by the products of tubercle bacilli liberated in vitro (tuberculin) but apparently not liberated in appreciable amounts in vivo, desensitization with these products (tuberculin) presents a fascinating problem, the exact nature and significance of which will have to be disclosed." The evidence questions whether the active constituent of in vitro natural filtrate from the growth of tubercle bacilli is liberated in vivo. In spite of this, desensitization of bacillary-tuberculo-allergically sensitized animals can be accomplished by appropriate treatment with tuberculin, so that animals thus prepared do not show a local specific skin reaction to tuberculin and are likewise protected against a lethal general allergic shock intoxication with tuberculin. The protective materials are contained in the blood of desensitized animals, and this protection can be transferred to a tuberculo-allergic-sensitive animal with the blood from a desensitized tuberculo-allergic animal. Tuberculo-desensitization with primarily nontoxic amounts of tuberculin exerts no beneficial or detrimental effect upon tuberculosis or upon specific tuberculo-

immunity. By 1942⁸ we began to see definitely the fallacy of tuberculin therapy for treating tuberculosis and also began to understand more fully the desensitization or immunization with tuberculin against tuberculin allergic intoxication as an extraneous phenomenon to tuberculosis and of no practical import. One year later⁹ it was noted that tuberculin therapy in man is not warranted by any evidence available at present from animal experiments. Then it was recognized that while we could desensitize against tuberculin-allergic intoxication with tuberculin, this exerted no appreciable effect upon the resulting tuberculosis, and the reaction to viable tubercle bacilli was not retarded. We were able to define two separate types of tuberculo-allergic hypersensitiveness: (1) that elicited by tuberculin, which seems to be of no practical importance in tuberculosis as it occurs naturally, and (2) that elicited by viable tubercle bacilli and which naturally assumes importance in the disease. These experiments clarify the reasoning of investigators in the past who assumed that tuberculo-allergy and tuberculo-immunity were synonymous or that they represented closely related phenomena. The deductions were not correct, however, since they were drawn from the use of tuberculin as the test agent for allergy. To complete the tuberculin picture, in 1943¹⁰ it was shown further that passive transfer of blood of tuberculin-immune guinea pigs to normal animals prevented tuberculin sensitization to tuberculin anaphylactic shock, and that passive transfer of blood of immune or anaphylactic-hypersensitive guinea pigs with or without the addition of tuberculin failed to protect tuberculin-hypersensitive guinea pigs from the effect of the lethal tuberculin anaphylactic shock.

SPECIFIC TUBERCULO-IMMUNITY

Our close association with specific tuberculo-immunity began in about 1935.¹¹ Without fully understanding avirulent tubercle bacilli, we were able to satisfy ourselves of the significance and potency of specific tuberculo-immunity by 1936.¹² Then it was shown for the first time that very small numbers of virulent tubercle bacilli mixed with large amounts of fine suspensions of avirulent tubercle bacilli would produce tuberculosis unhampered in the guinea pig, that tubercle was a function of dead bacillary bodies, and that viability played no decisive part in this except in so far as bacillary bodies were produced. We noted also that resolution occurred with tubercles formed by avirulent bacilli, and that previous injections of avirulent human and bovine tubercle bacilli retarded the subsequent infections with virulent human or bovine tubercle bacilli, while heat-killed avirulent and virulent bacilli exerted no such effect. Avirulent human and bovine tubercle bacilli were proved to produce no progressive lesions in man, and the bacilli lost their viability in these lesions in about six months. The reactions to viable avirulent tubercle bacilli in man showed a sequence of changed reaction upon repeated injections at definite intervals similar to the changes noted in animals and occurring coincidentally with the development of artificial immunity in these animals.¹³ It was pointed out that there is an apparent paradox resulting from this reaction in that the lesions produced by small and large numbers of virulent tubercle bacilli are retarded by the immune reaction as a result of preventing or retarding the multiplication of

virulent bacilli—*less lesion to virulent infection*; and, on the other hand, there is a greater tissue reaction in the immune organism as compared with the non-immune to large numbers of avirulent bacilli incapable of multiplying in the tissues—*more tissue reaction to avirulent bacilli*. Thus, conclusions drawn from pathologic findings alone, without knowing the quantity of either one of these two decisive factors concerned and necessary for interpretation (the number of bacilli producing the reaction or the existing immune condition present), can be of no practical value. In 1937¹⁴ we became more fully aware that these two reactions were separate entities, one which is the expression of a specific tuberculo-immunity and the other, still vague, which we erroneously referred to as “a type of protein intoxication” that does not become evident until large amounts of tubercle bacilli are either injected or disseminated internally after a primary contact with viable tubercle bacilli. We still referred to this as anaphylactic shock or reactions at that time. By 1938¹⁵ we became more confident of the lack of multiplication of avirulent tubercle bacilli and talked about the toxicity of these bacilli and the absence of infectivity with the possibility of resolution following the intravenous injection of excessive amounts into normal nonimmune guinea pigs. This led to our definition of an avirulent tubercle bacillus on the basis of the lack of ability to multiply in a highly susceptible host when injected intravenously. The property of avirulence and virulence of mammalian tubercle bacilli was consistently stable within a reasonable time, many months at least, on artificial mediums or in the animal body when properly tested.¹⁶ Changes in virulence of mammalian tubercle bacilli appear to be a gradual transition and usually occur only slowly over many generations of progeny of a pure strain. No guaranteed means of a ready or permanent transition appears to be available as yet. By 1939,⁴ we stated that “There is a striking quantitative difference between the specific immune and the concomitant allergic or anaphylactic features of tuberculosis which will eventually have to be given independent consideration in the complete evaluation of tuberculosis in man, since one is protective and the other shows a peculiar type of still unsolved intoxication.” The following year¹⁷ we said that the bacillary body sensitizes primarily to tuberculo-allergy, while the natural filtrate containing tuberculo-protein sensitizes to anaphylaxis and provokes anaphylactic shock and allergic intoxication but does not sensitize to allergy nor specifically immunize against virulent infection. At that time we concluded that tuberculo-anaphylaxis, tuberculo-allergy, and tuberculo-immunity were separate and apparently unrelated biologic phenomena. We were now able to demonstrate⁵ that the skin (allergic tuberculin) reaction may be entirely absent in animals which still retain their specific tuberculosis immunity, and vice versa, and that desensitization with tuberculin to the skin tuberculin reaction exerts no influence upon the specific tuberculosis immunity. We were also well aware at this time that the specific immunity persisted unabated in its effects for well over two years.¹⁸ In 1943¹⁹ it was noted that “In no disease are the relative amounts of interacting factors of more decisive significance than they are in tuberculosis; and yet no exact scientific measure of the interacting relations of causative agent and host exists which can be applied readily in evaluating these factors in man.” As a result,

there are those who, even to the present time, believe in an increased susceptibility following a primary infection, and this, a priori conclusion, is based on the fact that in some cases the disease progresses to an advanced stage. They fail to recognize the factors so involved and especially the possibilities in the fate and circumstances of heavily infected foci. No immunity is or can be absolute. It has its quantitative limitations, as it does in tuberculosis also. However, specific tuberculosis immunity shows definite quantitative aspects as gauged by graded virulent infecting injections and graded specific immunizing vaccinations with viable avirulent tubercle bacilli. In concluding the consideration of specific tuberculo-immunity, it might be added further that, unlike the tuberculin phenomenon, it has not been possible in our experiences to transfer passively specific tuberculo-immunity perceptibly to normal recipients.²⁰ Likewise, the specific immunity is not passed in utero from mother to offspring.²¹ Thus, immunization must be active to be appreciably effective for the recipient.

SPECIFIC TUBERCULO-ALLERGY

In tuberculosis, we are dealing with a disease of conspicuously dual aspects, specific tuberculo-immunity and a specific tuberculo-allergy, aside from the usual bacillary and pathologic anatomic interminations. We could not dwell long upon vital interpretations in tuberculosis without being confronted by the phenomenon of tuberculo-allergy. We were aware that intoxication in full-blown disease, as in many destructive diseases, may be associated with host tissue destruction and the absorption of disintegration products, but we also knew that normal animals could tolerate inconceivably large amounts of viable avirulent tubercle bacilli intravenously without violent response. Tuberculin apparently was not liberated in the host body and, even if it had been, desensitization or immunization to tuberculin was possible and did occur according to reliable scientific experimental test. We were aided by the preparation of purer tuberculins and bacillary suspensions in the solution of this problem. Our conceptions first began to take shape in 1940¹⁷ with the recognition that tuberculo-anaphylaxis, tuberculo-allergy, and tuberculo-immunity showed distinctive characteristics, pointing to their being separate and apparently unrelated phenomena. At that time it was noted that bacillary-sensitized or tuberculous animals do not respond to an injection of tuberculin after an appropriate incubation period by an acute anaphylactic shock reaction but do reveal a protracted (allergic) intoxication to a suitable amount of tuberculin with fatal issue after from one to three days except when the tuberculous involvement is very marked, when it occurs more rapidly. Also, tuberculin-sensitized animals do not develop an acute (anaphylactic) shock after the injection of young washed tubercle bacilli, while bacillary-sensitized and tuberculous (allergic) animals succumb to these injections. The bacillary and tuberculous (allergic) principals are never passively transferable to normal recipients. The lethal effect (allergic) of tuberculin in tuberculous animals appears to depend upon the time and amount of involvement following infection with virulent tubercle bacilli. Peculiarly enough, while it is possible to desensitize to tuberculin-allergic intoxication by means of tuberculin and also thus abolish

the local skin reaction (tuberculin reaction), this does not alter the specific immunologic condition appreciably nor does it prevent bacillary intoxication.⁵

In 1942²² a rather interesting set of experiments was performed in which it was shown that the addition of certain paraffins markedly accentuated the property of inducing allergic hypersensitiveness to tuberculin by heat-killed tubercle bacilli, verifying earlier work by Saenz, but these paraffins exerted little effect in accentuating the specific tuberculo-immunity produced by tubercle bacilli either dead or alive. In 1943⁹ we became more fully aware of two types of tuberculo-allergic hypersensitiveness: (1) that elicited by tuberculin, seemingly of no practical import, and (2) that elicited by viable tubercle bacilli, important naturally in the disease. It was noted also at this time that preliminary attempts to desensitize or immunize against the bacillary allergic hypersensitiveness by using dead or viable tubercle bacilli failed. In 1943²¹ it was noted that the specific tuberculo-allergic hypersensitiveness to tuberculin was not passed from mother to offspring in utero nor could it be transferred passively by intravenous injection of blood.²⁰ Before passing on to the implication of specific tuberculo-bacillary allergic hypersensitiveness in man, I will summarize our final attempt (in 1944²³) to desensitize against this condition in animals. Repeated intravenous injections of viable avirulent tubercle bacilli resulted in no appreciable reduction of hypersensitiveness either to tuberculin or to viable bacillary bodies but rather in an accentuation of the allergic hypersensitiveness; likewise, an attempt to desensitize tuberculo-bacillary-hypersensitive animals by means of repeated subcutaneous injections of heat-killed tubercle bacilli in mineral oil resulted in only a slight accentuation of the allergic hypersensitiveness. Dead (heat-killed) tubercle bacilli suspended in saline and subcutaneously injected repeatedly showed no appreciable effect on the tuberculo-bacillary allergic hypersensitiveness; however, when injected intravenously, a depression of the cutaneous allergic hypersensitiveness to tuberculin and bacillary bodies occurred (believed due to effect on general vascular or inflammatory response of animals). Dead avirulent tubercle bacilli repeatedly injected intravenously revealed no appreciable effect on the development of the tuberculous disease resulting from subcutaneous infection with highly virulent tubercle bacilli, but these same injections appeared to exert a slight detrimental effect on the specific immunity resulting from vaccination with viable avirulent tubercle bacilli which may be accounted for by the ability of the dead bacillary bodies to produce tubercles and productive tissue reactions.

Since 1918, it has been our custom to study carefully tubercle bacilli freshly isolated from human sources to determine their virulence. In 1942²⁴ we had an unusual opportunity to observe a classical example of death due to tuberculo-allergic bacillary hypersensitiveness in a patient who, at the time of death, harbored only avirulent tubercle bacilli. The spread of these bacilli resulted in the fatal tuberculo-allergic bacillary intoxication, a protracted death characterized by the same symptoms noted for such death in experimental animals. Post-mortem examination established the anatomic diagnosis of fibrocavernous tuberculosis in both lungs. The heart showed no abnormalities beyond a slight right hypertrophy. The significant pathologic changes were a pronounced marginal acute hyperemia

in the left lung not, however, attaining a pneumonic stage. This was the early result of a bacillary allergic reaction, the intoxication resulting in the collapse and death in a patient harboring only avirulent tubercle bacilli in the lungs in sufficient amount to cause the terminal lethal allergic intoxication. This patient with avirulent tubercle bacilli had what appears to be a clear-cut case of final lethal tuberculo-allergic bacillary intoxication in the human being, a condition which may arise in any active open case of tuberculosis. As the disease advances, the bacilli become available in sufficient amount for a sudden massive mobilization toward actively absorbing or reacting sites. To counteract this, we have as yet found no immediate treatment, although all abolition or collapse measures used at present serve as preventives against these accidents of tuberculosis.

SUMMARY

1. We have verified and demonstrated the following:

(a) A tuberculin anaphylactic hypersensitiveness exists and is produced by tuberculo-proteins (tuberculin) appropriately injected. It does not appear to be of any significance in the natural picture of tuberculosis in man or animals and is mainly of academic interest.

(b) Tuberculin (tuberculo-protein) anaphylactic hypersensitiveness can be transferred passively with the blood and is passed from mother to offspring in utero.

(c) Tuberculo-anaphylactic shock and tuberculo-allergic shock produced by the injection of tuberculin (tuberculo-protein) present entirely different clinical and pathologic manifestations and can be demonstrated in the same animal under appropriate conditions.

2. We believe that we have verified and demonstrated the existence of a relative specific tuberculo-immunity produced by (a) viable (avirulent human and bovine) tubercle bacilli, but not by (b) heat-killed nonviable tubercle bacilli by (c) the filtrate from growing tubercle bacilli on a nonprotein synthetic medium or by (d) tuberculo-proteins (tuberculins).

This specific tuberculo-immunity must be produced actively and persists over four years; it cannot be transferred passively with the blood; and it does not pass from mother to offspring in utero.

There is no means of measuring the specific immunity at present except by virulent infection.

3. We have verified and demonstrated the existence of a specific tuberculo-allergy which is produced by (a) viable tubercle bacilli and by (b) heat-killed nonviable tubercle bacilli which are more efficient when suspended in paraffin or mineral oils.

This specific tuberculo-allergy appears to be no measure of immunity, although it may be produced during infection coincident with it. When produced by avirulent tubercle bacilli, tuberculo-allergy reaches a maximum at about two months and may disappear in about six to twelve months. It cannot be transferred passively with the blood nor is it passed from mother to offspring in utero.

Desensitization or immunization against allergic tuberculin hypersensitivity can be achieved by appropriate injections of tuberculin.

Allergic bacillary hypersensitivity is not affected by tuberculin injections, so that tuberculin therapy appears futile for this purpose practically.

Specific tuberculo-immunity and specific tuberculo-allergy play a significant though individual part in the picture of clinical tuberculosis.

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LABORATORY METHODS

A RAPID TEST FOR DISTINGUISHING HUMAN FROM COW'S MILK BASED UPON A DIFFERENCE IN THEIR XANTHINE OXIDASE CONTENT

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HUMAN milk is purchased and distributed for infant feeding by many institutions throughout this country. The price paid to mothers for their milk is about fifteen times the cost of an equal amount of cow's milk. Thus, the dilution of human milk with cow's milk becomes a profitable procedure, and one which must be guarded against by the purchaser. We present here a simple test for detecting the admixture of human and cow's milk. It is based upon the fact that human milk is practically devoid of the enzyme xanthine oxidase, while cow's milk, even after commercial pasteurization, is rich in this enzyme. The xanthine oxidase activity of a milk sample can, therefore, be used as an index to its origin.

EXPERIMENTAL

A comparison of the xanthine oxidase activity of human and cow's milk was first made using a manometric procedure. The oxygen consumption of samples in the presence of hypoxanthine as a substrate was followed in the manner employed by Ball.¹ The test was arranged as follows: 2 c.c. of milk and 1 c.c. of 0.1 M phosphate buffer, pH 7.2, were placed in the main chamber of a conical Warburg manometer flask. The side arm of this flask contained 0.2 c.c. of a 0.05 M solution of hypoxanthine dissolved in 0.05 M NaOH. After temperature equilibration of the flasks in the water bath which was maintained at 37° C., readings were taken for a five-minute period in order to be certain that no oxygen consumption occurred in the absence of the substrate. The substrate was then added from the side arm, and readings were made every ten minutes for a total period of one-half hour.

The results of the analyses of twenty-six different samples of human milk and six different samples of cow's milk are shown in Table I. The samples of human milk were all unpasteurized and obtained fresh from the Mothers' Milk Directory at the Boston Lying-In Hospital.* The samples of cow's milk were purchased on the open market in Boston and represent milk from six different dairies. All samples were stated to be pasteurized.

The results clearly indicate that there is a marked difference in the xanthine oxidase activity of cow's and human milk. Only three of the twenty-six samples of human milk showed any measurable oxygen consumption, and in these the

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*Courtesy of Dr. F. J. Stare.

TABLE I. COMPARISON OF THE XANTHINE OXIDASE ACTIVITY OF HUMAN AND COW'S MILK

SAMPLE	COW'S MILK	HUMAN MILK
1	64	3
2	128	4
3	53	5
4	62	0*
5	84	0*
6	90	0
7 to 26		0

Results are expressed as cubic millimeters of O_2 consumed in thirty minutes at $37^\circ C.$ and pH 7.2 by 2 c.c. of milk with hypoxanthine as substrate.

*Colostrum.

amount of oxygen consumed was insignificant in comparison to that observed for cow's milk. For all practical purposes, it may be said that human milk is devoid of xanthine oxidase. The variation in activity shown by the different samples of cow's milk may be, in part, due to differences in pasteurization techniques at the various dairies. However, it has been reported² that the xanthine oxidase activity of an individual cow's milk is quite variable from day to day.

The xanthine oxidase activity of a milk sample may thus be used as an index to its source. If a sample of milk reputed to be human shows an appreciable xanthine oxidase activity, it is extremely likely that some cow's milk is present. The manometric procedure outlined may be employed for the purpose of assaying the xanthine oxidase activity of milk samples. A simpler method, however, is one based on the reduction of methylene blue.

The apparatus necessary to measure xanthine oxidase activity by the methylene blue method consists of ordinary pipettes, Thunberg tubes, and a water suction pump. The solutions necessary are (a) 0.005 per cent methylene blue solution and (b) 0.05 M xanthine or hypoxanthine in 0.05 M sodium hydroxide. The test is just about twice as rapid if hypoxanthine is available* for use.

To perform the test, 2 c.c. of milk are placed in the Thunberg tube† and 0.5 c.c. of the methylene blue solution is added. In the side arm or hollow stopper of the Thunberg tube is placed 0.3 c.c. of the xanthine solution or 0.2 c.c. of

TABLE II. REDUCTION OF METHYLENE BLUE BY MIXTURES OF HUMAN AND COW'S MILK WITH XANTHINE AS SUBSTRATE

PERCENTAGE COW'S MILK IN MIXTURE	METHYLENE BLUE DECOLORIZATION TIME (MIN.)	
	18° C.	23° C.
100	14	10
50	15	11
33	17	12
25	21	15
20	22	16
16	25	18
14	28	20
0	∞	∞

*Hypoxanthine is not available on the market at present, but xanthine may be purchased from Eastman Kodak Co., Rochester, N. Y.

†If Thunberg tubes are not available, a fairly satisfactory test may be run by employing ordinary test tubes containing from 2 to 3 c.c. of mineral oil. All solutions are pipetted into the tube under the oil and carefully mixed. The layer of oil serves to prevent access of air which would reoxidize the leucomethylene blue as it is formed.

REAGENTS

1. Citrate buffer, pH 4.9. Dissolve 18.9 Gm. of citric acid in 500 c.c. of water and add 180 c.c. of N. NaOH and 100 c.c. of N/10 HCl. Make up to 1 liter. (pH of buffer should be checked.)
2. Substrate, 1 per cent disodium phenyl phosphate (keeps about two weeks in icebox).
3. Dilute phenol reagent (Folin & Ciocalteu). Dilute 1 volume with 2 volumes of water.
4. 20 per cent sodium carbonate.

METHOD WITHOUT SUBSTRATE CONTROL

BLANK		TEST	
9 c.c. buffer		9 c.c. buffer	
1 c.c. substrate		1 c.c. substrate	
.5 c.c. serum		.5 c.c. serum	
Immediately add 4.5 c.c. phenol reagent		Incubate one hour at 37° C.	
Filter		Add 4.5 c.c. phenol reagent	
		Filter	
BLANK		TEST	
10 c.c. filtrate		10 c.c. filtrate	
2.5 c.c. 20 per cent Na_2CO_3		2.5 c.c. 20 per cent Na_2CO_3	
Incubate all at 37° C. for five minutes and read immediately		STANDARD	
		10 c.c. standard	
		2.5 c.c. 20 per cent Na_2CO_3	

The standard solution may or may not be necessary, depending on whether a calibrated photometer is available. The 10 c.c. standard solution should contain .04 mg. of phenol, 3 c.c. of phenol reagent, and water up to 10 c.c.

The milligrams per cent of phenol found in the blank subtracted from the milligrams per cent of phenol found in the test give the milligrams per cent of phenol presumably split by the enzyme and are considered "King units" of activity.

We employed this method for some time and obtained what appeared to be clinically reliable results. However, when a new supply of disodium phenyl phosphate was used, we suddenly began to get high results almost routinely. Further investigation of this difficulty led to a still more improved procedure which we are at present employing.

We discovered that upon incubation of substrate alone (disodium phenyl phosphate*) definite values of phenol were hydrolyzed.† This may be explained by the action of an acid medium or by the presence of impurities in the substrate. In any case, such a value must necessarily be deducted from the test regardless of what causes this hydrolysis. This necessitated a second blank made up of substrate and buffer in addition to the original blank. The latter two results are added together and subtracted from the test.

*Obtained from Paul-Lewis Laboratories, Inc., Milwaukee, Wis.

†Gutman and Gutman recommend the use of a highly purified phenyl phosphate. Such a reagent is not commercially available. In view of the great clinical importance of the test, it is desirable to have a method which can be accurately performed even in smaller hospitals which do not have the facilities for checking or improving the purity of commercial phenyl phosphate. The technique presented in this paper was devised for this purpose and appears to be the most practical way of overcoming the above difficulties.

IMPROVED PROCEDURE

B ₁ (SERUM BLANK)	B ₂ (SUBSTRATE BLANK)	TEST
--	9 c.c. buffer	9 c.c. buffer
10 c.c. water	.5 c.c. water	--
--	1 c.c. substrate	1 c.c. substrate
.5 c.c. serum	--	.5 c.c. serum
Immediately add 4.5 c.c. phenol reagent	Incubate one hour at 37° C.	Incubate one hour at 37° C.
Filter	Add 4.5 c.c. phenol re- agent	Add 4.5 c.c. phenol re- agent
	Filter	Filter

Take 10 c.c. of filtrate from B₁, B₂, Test, and Standard if necessary (make up as previously described). The color is developed by the addition of sodium carbonate and incubation as described in the former method. The milligrams per cent found in B₁ plus B₂ subtracted from the milligrams per cent of phenol found in Test give the milligrams per cent of phenol split by acid phosphatase, which become "King units" by definition. We have had very good results employing a Cenco photometer which we calibrated for phenol.

DISCUSSION

The results in Table I show that larger values are obtained by the method without substrate control. This is of special interest because most of the values are above the original limit of normal values published, 1 to 4 King units. By the improved procedure, much smaller figures are realized. The values do not seem to run above 4 except when pathology is found, as shown by Table II. Only two patients of eleven revealed no immediate evidence of carcinoma of the prostate.

TABLE I

CASE	VALUES OBTAINED WITHOUT SUBSTRATE CONTROL	IMPROVED PROCEDURE
	(KING UNITS)	(KING UNITS)
1	6.6	2.1
2	3.5	0.15
3	0.9	0.3
4	3.9	0.9
5	4.8	2.7
6	4.2	0.9
7	4.8	0.3
8	3.6	0.3
9	4.2	0.9
10	6.3	3.3
11	10.8	2.4
12	8.4	0.6
13	8.7	0.6
14	9.9	1.2
15	9.0	0.0
16	8.4	0.3
17	10.2	2.1
18	10.8	0.6
19	12.6	3.3
20	3.9	0.6
21	6.6	0.6
22	13.8	3.3

scopic fields are examined before a negative report is given for a sputum specimen.

Careful counts of the organisms per microscopic field were made in most instances. This was not done when the count per field was over 100. For counts between 100 and 300, the figure recorded was a careful estimate. Three hundred represented the maximum count recorded for any field. When the count per microscopic field was from one to fifty organisms, from five to twenty-five fields were counted and the average per field determined. The primary objective was a fair comparison between the methods employed.

Findings were recorded as positive when at least three typical acid-fast organisms were found by one or more methods. If less than three were found, another specimen was requested. The goal in this study was set at 100 positive specimens in order that simple percentage determinations could easily be made in comparing the efficiency of the various methods.

OBSERVATIONS

Of 397 specimens examined, 283 were found negative by all methods, 100 were positive by one or more methods, and fourteen yielded less than three typical acid-fast organisms by the three methods combined. For each of the latter specimens, another specimen was requested.

The specimens found positive are of particular interest for purposes of comparison. Most specimens were sent to the laboratory for either diagnosis or a check on treatment. Eighty-four per cent of the specimens found positive were examined to aid in diagnosis and 12 per cent to check on treatment; for 4 per cent the purpose was not designated.

Seventy-seven of the 100 positive specimens were found positive by all of the methods used. The other twenty-three were found positive by one or two methods. These observations are given in Table I.

TABLE I. NUMBER OF SPECIMENS FOUND POSITIVE FOR TUBERCLE BACILLI BY METHODS INDICATED

METHOD	NUMBER	PER CENT
All methods used	77	77
Clorox and NaOH-alum only	12	12
Clorox only	6	6
Clorox and direct only	3	3
NaOH-alum and direct only	2	2
NaOH-alum only	0	0
Direct only	0	0
Total	100	100

From this table it may be seen that acid-fast organisms were found in 98 per cent of the 100 positive specimens by the clorox method, 91 per cent by the NaOH-alum method, and 82 per cent by the direct smear.

In Table II, it may be seen that the average count of organisms per microscopic field by the clorox method was unexceeded for 81 per cent of the specimens. The NaOH-alum method yielded an unexceeded count for 24 per cent of the specimens and the direct for 10 per cent.

A far more striking comparison for the three methods is observed in the

TABLE II. FREQUENCY OF HIGHEST COUNT OF TUBERCLE BACILLI PER MICROSCOPIC FIELD FOR POSITIVE SPECIMENS

METHOD	PER CENT
Clorox	68
NaOH-alum	13
Direct	6
Clorox and NaOH-alum	9
Clorox and direct	2
Clorox, NaOH-alum. direct	2
Total	100

average number of acid-fast organisms found per 100 microscopic fields. This comparison is based upon the seventy-seven specimens which were found positive by each and all of the three methods used.

The large number of fields counted by the direct method is due to the fact that it was necessary to examine more fields to find acid-fast organisms by this method.

DISCUSSION

The results obtained show the same trend as have been shown in similar previous studies.^{1, 2} For 188 specimens found positive in the combined studies made in this laboratory by the clorox method and by the NaOH or NaOH-alum method, the average number of organisms per 100 microscopic fields was 1,274 for the clorox method and 438 for the NaOH methods. The average count of 1,052 recorded in Table III for the clorox method as compared with 465 for the NaOH-alum method per 100 microscopic fields compares favorably with the over-all picture found in these studies.

TABLE III. AVERAGE NUMBER OF ACID-FAST ORGANISMS PER 100 MICROSCOPIC FIELDS FOR SEVENTY-SEVEN SPECIMENS POSITIVE BY ALL METHODS

METHOD	FIELDS COUNTED TO FIND SPECIMENS POSITIVE	TOTAL ORGANISMS FOR FIELDS COUNTED	AVERAGE ORGANISMS PER 100 FIELDS
Clorox	855	8,996	1,052
NaOH-alum	967	4,492	465
Direct	1,406	3,144	224

The fact that an average of 1,052 organisms per 100 fields was found in this study by the clorox method as compared with 1,274 by the same method for the combined studies seems to indicate that fewer organisms were present. In that case the increase in the average, 465 for the NaOH-alum method as compared with 438 for the combined studies by the NaOH methods, becomes somewhat more significant. The comparative increase in efficiency of the NaOH digestion method in this study is probably due to the use of alum flocculation for concentration of the tubercle bacillus. This is in accord with the report of Hanks³ on the use of chemical flocculants as an aid in concentrating acid-fast organisms in laboratory specimens. However, the results still definitely favor the clorox method.

Acid-fast organisms were found in twelve of the 100 positive specimens by the NaOH-alum method in which they were not found by the direct smear. Eighteen specimens were found positive by the clorox method which were not

found positive by the direct smear. In six of the specimens found positive, tubercle bacilli were found only by the clorox method. None were positive by the NaOH-alum method only, but acid-fast organisms were detected in two specimens by the NaOH-alum and direct methods and not by the clorox method.

For finding some acid-fast organisms in 100 positive specimens, the clorox method proved 98 per cent efficient, the NaOH-alum method 91 per cent, and the direct smear 82 per cent efficient.

By the clorox method 68 per cent of the specimens found positive yielded the highest average count per microscopic field. By the NaOH-alum method 13 per cent and by the direct smear 6 per cent gave the highest average count per microscopic field.

In every comparison the clorox method gave more efficient results in this study than did the NaOH-alum method.

Because of the more complete digestion by the clorox method, the centrifuged sediment from positive specimens usually contained more organisms per given amount of sediment than did those obtained with the NaOH-alum method. As in previous studies when the clorox method was used, microscopic fields showed only acid-fast organisms and no visible extraneous material.

SUMMARY

1. Every comparison in this work shows that somewhat greater efficiency for concentrating tubercle bacilli in sputum was obtained by the clorox method than by the NaOH-alum method.

2. Both the clorox and the NaOH-alum method surpass the direct smear for finding acid-fast organisms in sputum.

3. Acid-fast organisms were found in more specimens by the clorox method than by the NaOH-alum method.

4. For identical positive specimens, more than twice the average number of acid-fast organisms were found per 100 microscopic fields by the clorox method than by the NaOH-alum method.

5. The clorox method is much simpler to handle than the NaOH-alum method and gives more complete digestion, resulting in less visible extraneous material in the microscopic field.

6. For specimens containing very few organisms, a large amount of specimen may be used for concentration by the clorox method without clouding the microscopic picture.

7. When the clorox method is used, the small cotton swab should be moistened in clear blood serum before transferring the sediment from the tube to the slide. This furnishes background and fixative when there are only a few acid-fast organisms present.

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A TRAP WITH HOLDER FOR HANDLING VICIOUS LABORATORY ANIMALS SUCH AS WILD RATS*

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THE apparatus herein described is the outgrowth of difficulty experienced in experimenting with the cotton rat (*Sigmodon hispidus*), which is not only difficult to handle, but also quite vicious, and cannot be managed like the ordinary laboratory animal.

The description of the holder employed in this apparatus is similar to the one described in a private communication† from which we quote:

To examine their blood we have devised a cylinder of $\frac{1}{2}$ inch mesh, about five inches long and from 1 to $1\frac{1}{2}$ inches in diameter, closed at the one end and covered with fly screening to prevent the rat from biting through the $\frac{1}{2}$ inch mesh. A piece of cardboard large enough to cover the opening of the cage should have a hole cut at the bottom to fit the open end of the cylinder. By blowing on the rat the animal will be induced to go into the cylinder. We use a cloth to hold the rat in the cylinder and find it easy to make an examination of its blood by clipping the tip of the tail.

On attempting to use this method with large individual cages, such as type L C-28‡ with sloping door, we were unable to get the animals to enter the above type of holder.

Our apparatus makes capture of the animal certain, so that it may easily be weighed, bled, or given oral or parenteral medication.

The apparatus consists of a cylindrical holder, a truncated cone-shaped tunnel or trap, and two comblike stops for immobilizing the captive. The trap is soldered to a metal shield which fits over the opening of and fastens to the animal cage. A trap door which closes the opening in the metal shield is operated by a spring catch. To the free end of the trap is soldered a metal flange over which the holder slips snugly when the animal is to be captured. The larger stop is used with the trap, the smaller with the holder.

Fig. 1 illustrates the various units of the apparatus.

SPECIFICATIONS

The shield to which the funnel is soldered is made of 22-gauge galvanized sheet iron of sufficient dimensions to overlap the open doorway of the cage. Ours is $7\frac{1}{2}$ inches high by 9 inches wide and fits well over Wahmann's standard cages L C-26 and L C-28, with openings measuring $6\frac{1}{2}$ inches high by $7\frac{1}{2}$ inches wide and $5\frac{1}{2}$ inches by $5\frac{1}{2}$ inches, respectively. The trap door is made

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‡George H. Wahmann Mfg. Co., Baltimore, Md.

of 22-gauge galvanized sheet iron and is held in place by a groove at either side of the opening in the shield. A spring catch is attached to the shield to operate the trap door.

A circular hole, $4\frac{1}{2}$ inches in diameter, is cut near the bottom along the width of the metal shield so that its center is midway between the sides, leaving approximately $\frac{1}{4}$ inch of sheet iron at the bottom for soldering. The base of the trap, which is about $\frac{1}{4}$ inch larger than the opening in the shield, is soldered over the opening.

The shield, with the trap attached, placed with its flat surface over the opening in the cage makes a perfect runway from cage floor to the holder when the trap door is open.

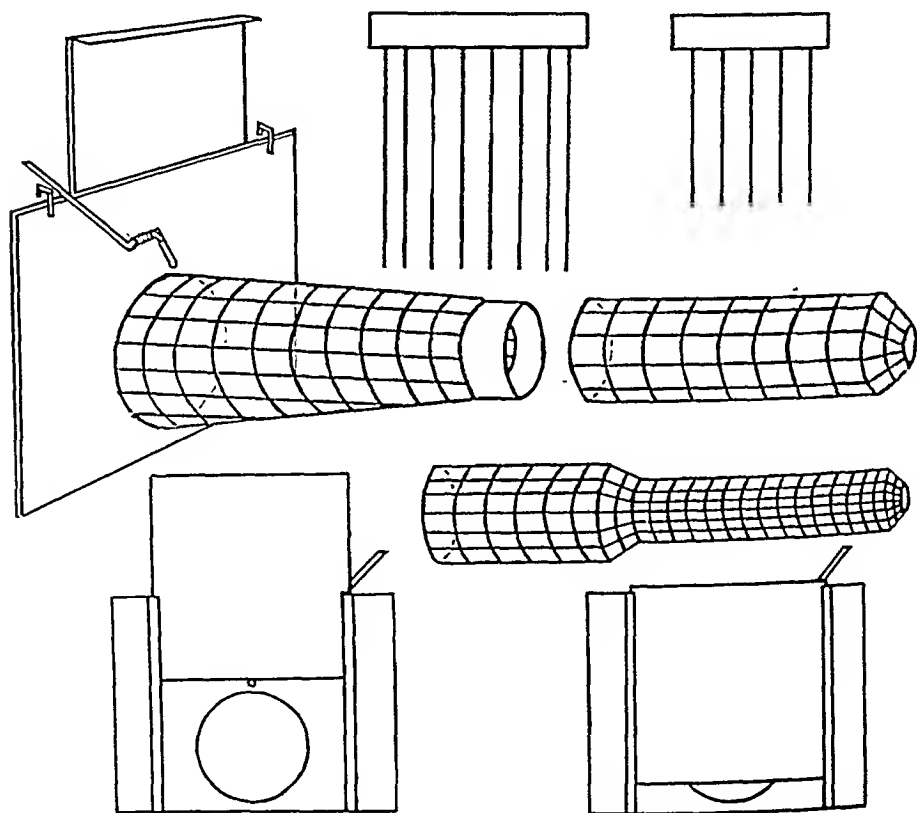


Fig. 1.

The shield is fastened to the cage by means of two pieces of No. 14 galvanized iron wire, each being approximately $2\frac{3}{4}$ inches long and fastened vertically to the upper edge of the front of the shield (the side to which the trap is fastened) about 1 inch from each end with approximately $1\frac{1}{2}$ inches being soldered to the base. They are then bent to a right angle over the edge of the base and again at a right angle about $\frac{3}{4}$ inch from each end thus making hooks that fasten over the upper framework of the cage, securely fastening it to the open doorway.

The trap is a truncated cone of 18-gauge, 4-mesh hardware cloth. It is $7\frac{1}{2}$ inches long with the inside diameter at the base being $4\frac{3}{4}$ inches and that at the top, $2\frac{1}{2}$ inches.

Soldered to the inner part of the small end to a depth of about $\frac{1}{2}$ inch is a galvanized sheet iron flange $1\frac{3}{4}$ inches long and made to taper slightly so that the free end measures about 2 inches outside diameter and over which the large end of the holder fits tightly.

The holder is made of hardware cloth similar to that used for the trap. It is a cylinder 9 inches long and $2\frac{1}{4}$ inches inside diameter. One end is slightly tapered $1\frac{1}{4}$ inches from the opening to form a circular opening $\frac{3}{4}$ inches in diameter and is made secure by a ring of wire soldered around the entire circumference. We also have other holders with inside diameters of $1\frac{3}{4}$ inches, $1\frac{1}{2}$ inches, and $1\frac{1}{4}$ inches. See Fig. 1 showing the method of reducing the diameter so that various-sized animals may be accommodated.

The stops are of two sizes but are of similar material. As mentioned, they are shaped like a comb, the base being made of $\frac{1}{16}$ inch by $\frac{3}{4}$ inch flatiron and the teeth of $\frac{1}{16}$ inch welding rods, which are soldered upon the flatiron.

The larger comb has a base four inches long, with eight rods $4\frac{3}{4}$ inches long soldered perpendicular to the base in parallel. Distance between rods is not equal but as follows: $\frac{1}{4}$ inch, $\frac{7}{16}$ inch, $\frac{1}{2}$ inch, $\frac{7}{16}$ inch, $\frac{7}{16}$ inch, and $\frac{1}{4}$ inch. The first and last rods are soldered about $\frac{1}{2}$ inch from each end of the base. The unequal distance makes it possible to thrust the rods through the mesh in the tapering cone when trapping the animal.

The small comb has a base $2\frac{1}{2}$ inches long with five rods $3\frac{1}{2}$ inches long soldered similar to the large one but beginning about $\frac{7}{16}$ inch from one end and spacing all $\frac{1}{2}$ inch apart, variability in this respect not being necessary since the holder in which it is used is a cylinder.

CAPTURING THE ANIMAL

To capture the animal, the apparatus is assembled as described. The shield with the open trap and holder attached is fastened to the open doorway of the cage. By blowing upon the animal from the rear of the cage, it will usually seek the opening and enter the trap. As soon as the animal enters the trap, the lever attached to the spring is moved by slight pressure of the forefinger which releases the trap door, thereby preventing re-entrance into the cage. The large stop may be used to induce the animal to move to the holder, and, as it enters, the smaller stop is similarly used, forcing the animal to the extreme tip where it is firmly held by the stop which has been successively moved behind it as it moves forward. The diameter of the cylinder is so small that the animal cannot turn around.

The holder containing the animal is then removed from the trap by slipping it off the smooth metal flange. The animal is not injured by the apparatus as it may be if tongs or similar instruments are used for catching and holding. The holder with the stop in place is simply turned over so that the flat side of

the stop is down while the animal is weighed. The flat surface prevents the apparatus from rolling off the balance.

Either the opening at the end of the holder or a mesh of the hardware cloth may be used to pass a tuberculin syringe through to feed the animal a definite amount of a substance. It is possible to inject an animal by various routes when it is in the holder or to take blood samples from the heart, tail, or ears, or by clipping off a toenail.

We feel that the apparatus described herein is especially convenient and useful for handling rodents which have a tendency to bite when picked up by hand.

A LABORATORY HOLDER FOR IMMOBILIZING EXPERIMENTAL RATS

ERNEST J. UMBERGER, M.A.

WASHINGTON, D. C.

IN THE pharmacologic study of ointments and other drugs in this laboratory, it was deemed desirable to study their effects on the albino rat. The difficulties of immobilizing these animals for extended periods of time to prevent contamination by licking and the eating of feces, and to prevent injury to the animals or to the site of application, were overcome by the design of a holder to be described. The usual animal board was found unsuitable for our purposes. Eleftheriou¹ described an apparatus in which the animals were placed into a cloth apron supported between two metal frames, and Leeloux² described a similar apparatus for mice. Neither of these, however, allowed for the feeding and watering of the animals during the period of confinement.

The holder herein described is easily constructed of readily available materials. Details of the holder are shown in Fig. 1. The neck and hip stocks were made of sole leather approximately $\frac{3}{16}$ of an inch thick, and the locks were tin plate cut from a tin can. The locks were secured at the hinge end with copper harness rivets and were fastened with small brass bolts $\frac{1}{8}$ of an inch in diameter and of suitable length. The dimensions shown are not critical but were found suitable for a rat weighing 250 grams. The stock openings can be made larger or smaller to accommodate the size of rat desired. The size of the holder shown has been used for smaller rats by padding the upper part of the openings with cotton wads held in place with adhesive tape. Care should be taken, however, that the space between the bottom of the opening of the hip stock and the floor of the cage be not too great or injury to the animal may result. The bars separating the stocks were made of brass rods $\frac{3}{16}$ of an inch in diameter threaded at each end. One end of each is threaded for two inches to allow for adjustment to the length of the body of the rat.

In use, the entire holder is fastened to the bottom of a regular wire-mesh individual steel cage by means of small 12-gauge steel wire hooks. In Fig. 2 is shown such an arrangement with an animal in place, the side of the cage being bent back for a better view. A battery of holders could be fastened to a board to enable simultaneous observation of the animals. Food and water can be supplied to the animals in the cages in the usual manner.

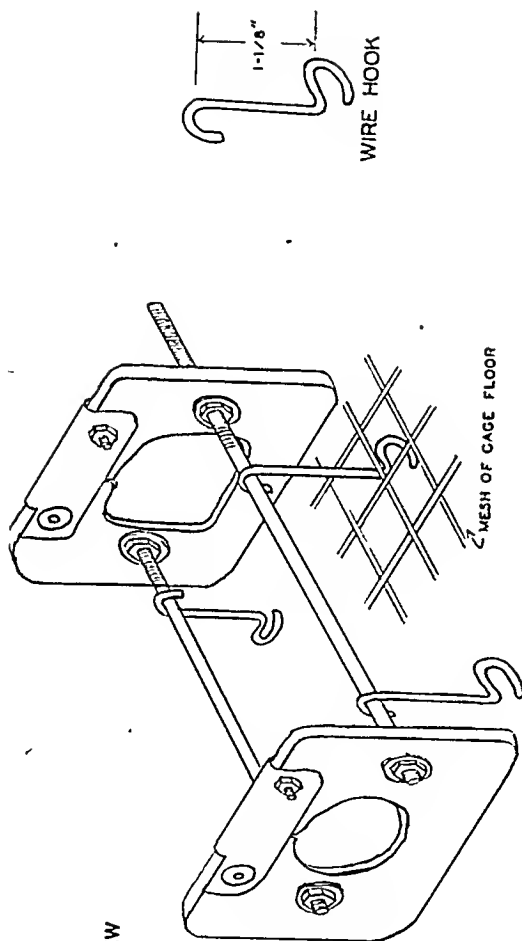
Although the rat can be put into the holder by two operators without anesthesia, the latter generally has been used. The animal is lightly anesthetized with ether and after opening the locks, the flexible leather stocks are bent aside to permit the entrance of the neck and hips. The locks are then closed by means of the bolts and the holder fastened to the bottom of the cage.

For most of the experiments, the animals were thus confined from one to twenty-four hours with a maximum of seventy-two hours. The animal rests

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GENERAL VIEW

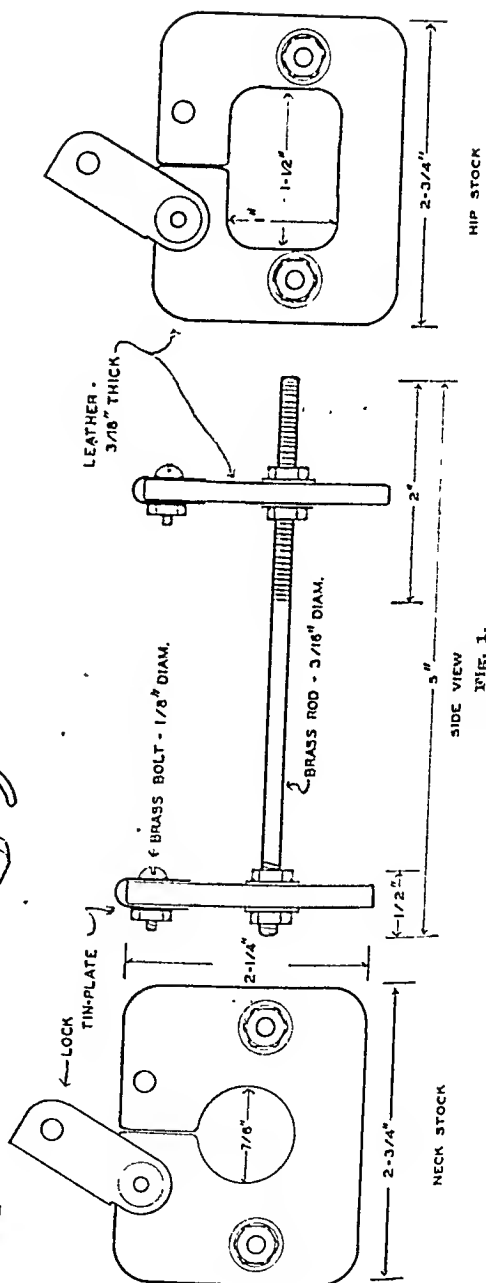


WIRE HOOK

1-1/8"

2. MESH OF CAGE FLOOR

DETAIL



LEATHER .
3/8" THICK

4

6 BRASS BOLT - 1/8" DIAM.

BRASS ROD - 3/16" DIAM.

NECK STOCK

SIDE VIEW

Fig. 1.

HIP STOCK

comfortably on the bottom of the cage, but occasionally the eyes of animal may appear congested due to struggling.

In order to gain some idea of the effect of confining the animals for periods as long as seventy-two hours, the following experiment was performed. Eight female rats averaging 250 grams were selected. All were lightly anesthetized with ether, placed in separate cages, and given a measured, ample amount of food and water. Four of the animals were confined in the holders. The animals were weighed daily. After seventy-two hours, the control rats had gained 2 per cent and the confined rats had lost 10.5 per cent of their initial weight.

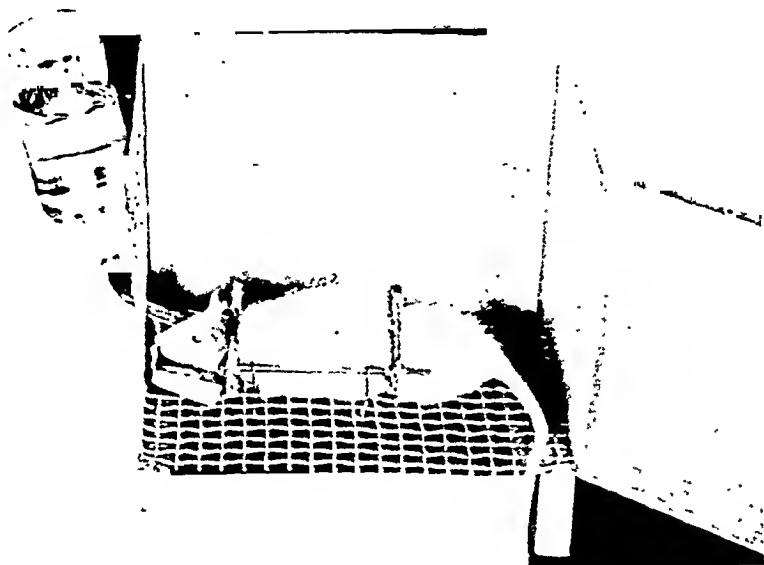


Fig. 2.

The confined animals were now removed from the holders and given ample food and water, while the animals which had been used for controls were deprived of all food and water. After an additional seventy-two hours (one hundred and forty-four hours from the beginning of the experiment), the control animals had lost 18.8 per cent of their weight, and the previously confined animals had gained 15.7 per cent, or a gain of 3.6 per cent over the one hundred and forty-four hour period. The animals appeared normal in every respect. It is apparent that while the animals are somewhat ill at ease while confined and lose some weight during that time, recovery is rapid and complete. The records show that the confined animals ate and drank about one-half as much as the control animals.

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2. Lécloux, L.: Appareil simple pour contention prolongée de petits animaux. *Bull. d'histol. appliq. à la physiol.* 4: 403, 1927.

A NOTE ON THE UNSUITABILITY OF GUINEA PIG JEJUNUM FOR STUDY BY THE MAGNUS METHOD

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BERNHEIM* has made the interesting and rather curious observation that pilocarpine will relax the contraction induced by histamine in isolated strips of the jejunum of the guinea pig.

We have found that records of the contractions of the jejunum of guinea pigs, made by the method of Magnus, indeed appear to show that marked relaxation of histamine spasm is produced not only by pilocarpine, but also by other parasympathomimetic agents; for instance, physostigmine and acetyl- β -methylcholine. However, if the intestine is observed rather than the recording lever, it is found that what is recorded as relaxation is actually a response to a powerful wave of contraction of the circular musculature of the jejunum, a phenomenon which actually lengthens the intestinal strip.

Further evidence that the apparent relaxation is artefactual was obtained by recording the changes in pressure which are produced within the jejunum by histamine and by pilocarpine. The proximal end of a strip of jejunum from 4 to 5 cm. was tied off. The distal end was tied to a small cannula, which was attached to a 1 c.c. pipette, graduated in 0.01 c.c. The jejunum was suspended in 50 c.c. of warm, oxygenated Ringer's solution. The pressure inside the intestine was recorded by noting the variations in level of the Ringer's solution within the pipette. A typical experiment showing maximal and minimal levels of the solution, as affected by histamine and by pilocarpine, is presented in Table I. (Since the pipette is read from 0 above to 1.00 below, a decrease in the readings accompanies a rise in the fluid column and therefore corresponds to an increase of pressure within the intestinal segment.)

TABLE I

	MAXIMAL LEVEL (C.C.)	MINIMAL LEVEL (C.C.)
Before addition of drug	0.77	0.79
After addition of histamine (1:50,000)	0.50	0.53
After addition of pilocarpine (1:50,000)	0.38	0.40

Thus, following the contraction induced by histamine, pilocarpine induces a further increase in the pressure inside the jejunum, even though the length of the intestinal segment may be increased.

It is concluded that the jejunum of the guinea pig, at least for some purposes, is quite unsuitable for studies by the method of Magnus. This deficiency is due to the presence of powerful circular muscle fibers, the contractions of which may overshadow contractions of the longitudinal fibers.

From the Wyeth Institute of Applied Biochemistry.

Received for publication, Nov. 19, 1945.

*Bernheim, F.: Interaction of Pilocarpine and Histamine on the Intestine, *J. Pharmacol. & Exper. Therap.* 43: 509, 1931.

PHOTOMICROGRAPHY: A SHORT METHOD USING NEGATIVE PROJECTION PRINTS

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THE pages of medical periodicals and textbooks testify to the educational value of photomicrographs in the demonstration of tissue structure and changes resulting from disease. Rarely, if ever, does the average physician think of preparing such prints for himself; however, in spite of the fact that the value of the photomicrograph is so generally understood, relatively few of the smaller clinical laboratories are prepared to make such prints for him. There are many reasons for this, the most obvious being the fact that a special type of fairly expensive apparatus and a complicated technique must be available. Such equipment, because of its limited usefulness in a small clinical laboratory, is usually considered a luxury and no photomicrography is therefore available. This difficulty will continue to exist as long as physicians adhere to the unreasonable belief that the finished photomicrograph must show the field *just as the pathologist sees it through his microscope*. It has been assumed that to be acceptable the end product must be a positive print made from a negative which has been exposed in a camera-like arrangement which holds the film in focus over the microscope. However, if it is remembered that the fundamental purpose of medical illustration is the clarification of points emphasized in the written text, it may seem to be quite legitimate to question the absolute need for adherence to this traditional form of photomicrography. It is conceivable that some other type of reproduction will make microscopic details just as clear to the reader.

Several months ago I was forced by the absence of any apparatus for standard photomicrography to make a decision on this point and to improvise a technique which, as results were reviewed, seemed to have several merits beyond an obvious simplicity. A case of fatal dissecting aneurysm of the thoracic aorta, of well-authenticated traumatic origin, came to autopsy at the Mary McClellan Hospital and, as a rare example of this disease, the case history was prepared for publication with a discussion of the literature. Severe histologic sections of the injured aorta were considered instructive enough to be used. Two photomicrographs were made from the stained slides in the form of negative paper prints by projection, and these hybrid photomicrographs proved acceptable as illustrations in a previously published article.*

In spite of the fact that it has been impossible to find any medical article using or describing negative projection prints of this type, no claim will be made that medical illustrators have never before considered the idea. If negative prints have been suggested, it is more than probable that medical authors

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*Leonard, D. W.: Dissecting Aneurysm of the Thoracic Aorta Due to Trauma, *Am. J. Surg.* 69: 344-351, 1945.

themselves have been the ones to discard what appeared to them to be distortions of real microscopic anatomy. If this has been the case, it is unfortunate that someone did not point out that whatever "distortion" resulted from the transposition of density values from positive to negative might have been improvements in the visualization of the tissue and that the change could not have been any more artificial than the already accepted artifacts of staining. The change might also have actually been more "true" in relation to living tissue than any other kind of photography.

MAKING PROJECTION PRINTS

Simplicity is the first advantage to be credited to a technique which makes prints on paper directly from the microscope slide. It is a darkroom technique but, in this respect, it is essentially the same as the step that has been used in the standard routine to get paper prints from negatives. It uses the slide in place of the negative and the microscope as the enlarger. Most teachers of pathology have at one time or another used a microscope as a means of projecting the enlarged image of a tissue section onto a screen for class demonstration. To make a projection print, it is necessary only to arrange the micro-

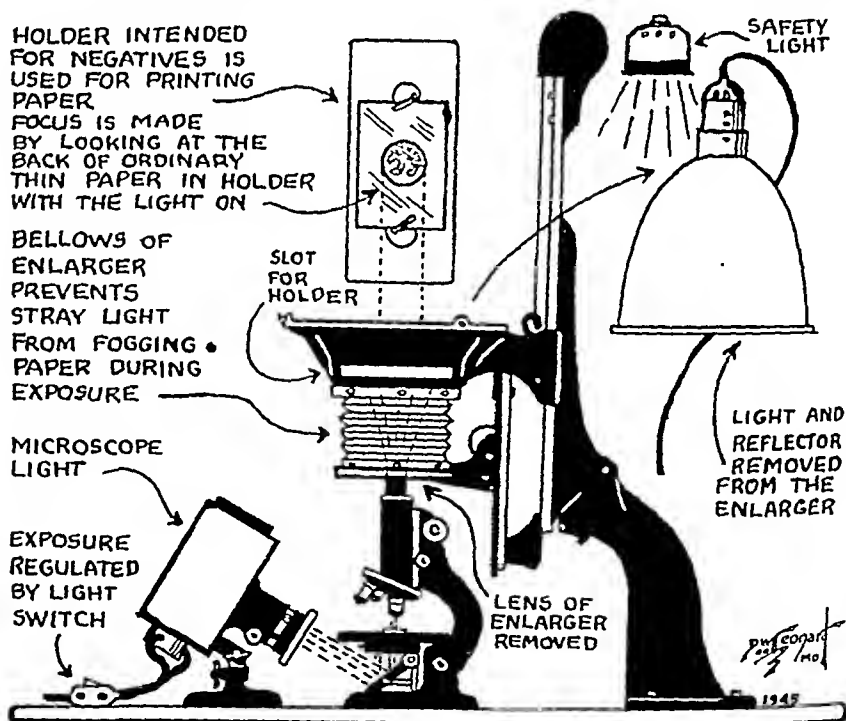


Fig. 1.—Assembly of apparatus used for taking the photomicrographs reproduced with this discussion. Although the image of a tissue section may be projected directly onto printing paper tacked to a board, it is safer to make use of some arrangement like the bellows of an enlarger as a light-trap to protect the paper from the stray light that will unavoidably leak from any microscope lamp. A commercial photographer may combine his enlarger with the doctor's microscope, if the latter cannot himself use this piece of photographic equipment.

scope, the slide, and the light source so that the image will be projected onto the photographic paper. For medical illustrations, a moderate enlargement is all that usually will be desired so that the suitable distance from the ocular of the microscope to the paper will seldom be more than a few inches. The entire assembly may be quite compact. The advantage of a scheme which obtains a finished picture in a single step will occur to anyone and the technical details of the process will be obvious to any physician having some knowledge of photography. It will not be necessary in this discussion to do any more than illustrate a simple arrangement of my equipment (Fig. 1) and to demonstrate by examples that the essential features of a tissue section can be shown as well in a negative print as in any other photomicrograph (Figs. 2 to 5).

When an individual has assembled a combination of his own available equipment, he will have to standardize certain values, the first being the strength of his light source as it effects the length of exposure which will be required for each magnification and for slides which vary in their staining. A few trial prints should establish these values. Projection printing paper registering various degrees of contrast may be tried, but sharp contrast paper (No. 3) with a glossy finish will usually be the choice. The framing of the pictures takes care of itself because the field selected will be framed automatically in the circle of the microscope lens. After the exposure has been made, the steps in development will be the same as those of normal photography.

INTERPRETATION

My experience with negative prints has already shown that the usual reaction of the medical observer is instant interest in the clear details and an attempt to identify the tissue while completely unaware of the reversal of tone values this type of photomicrograph presents. A pathologist's more discerning inspection, of course, discovers the reversal with involuntary resentment or curious pleasure, according to that individual's attitude toward innovations. For his benefit it may be argued that space is empty and dark and that a section of tissue may be mounted like a lace curtain in front of this empty background. The resemblance to objects viewed under dark field becomes striking and one may become enthusiastic with the realization that he has discovered a picture which appears just as he had imagined the original, unstained tissue section must have looked as it came off the microtome knife.

From such photomicrographs it should not be difficult to demonstrate the changes caused by disease and to correlate these changes with the descriptions recorded by those who have studied the same specimens directly under the microscope.

SUMMARY

Out of the need for self-made photomicrographs has developed a suggestion that any medical author can resort to the use of a negative projection print whenever he needs a photomicrograph for illustration. To the obvious advantage of requiring no more than the equipment already available can be.

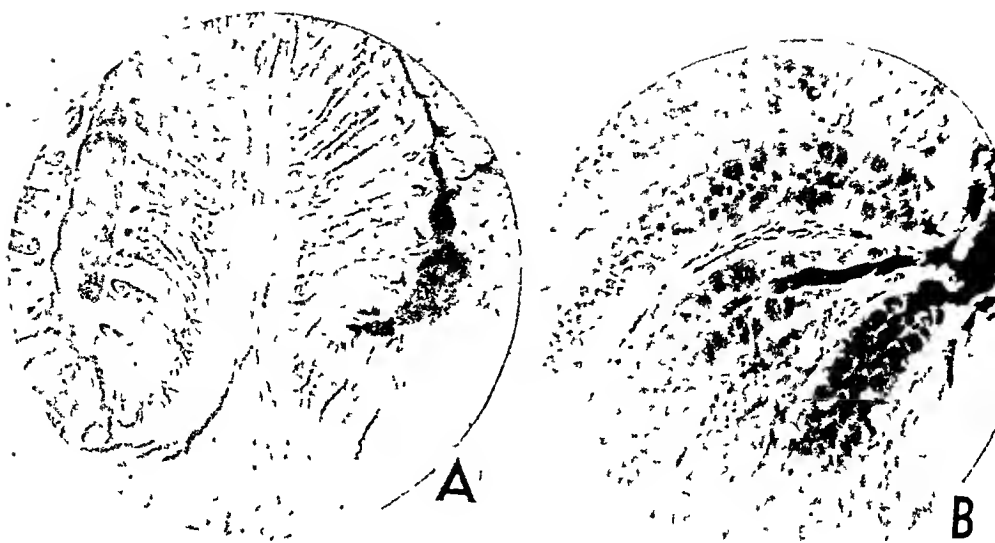


Fig. 2—A, Large intestine. Mucous, submucous, and muscular coats are all distinguishable. The crypts are for the most part cut longitudinally and the secreting elements are outlined in white by the cell nuclei near the basement membrane. B, Large intestine. High power magnification of the crypts to demonstrate the detail of the mucus containing goblet cells.

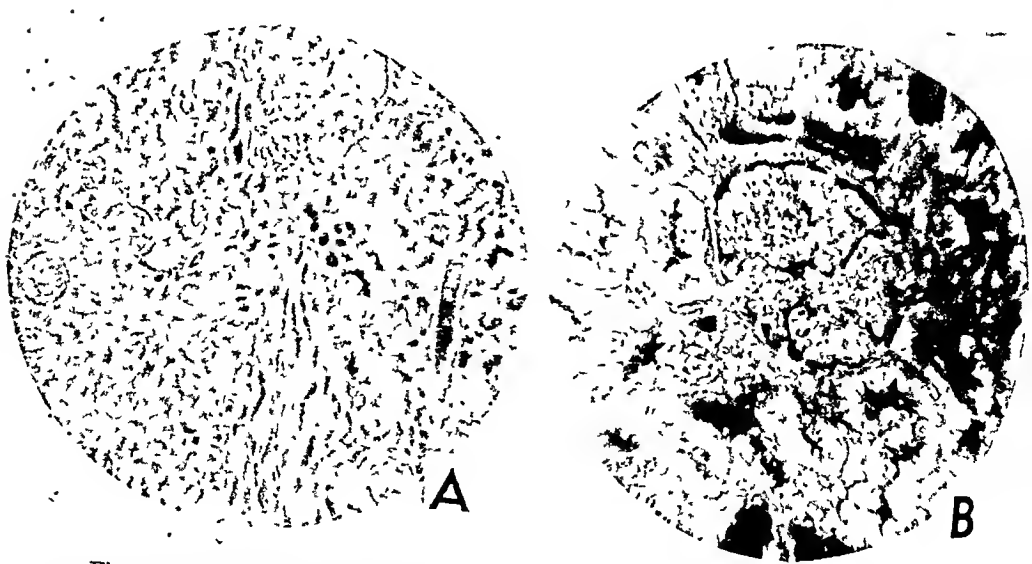


Fig. 3—A, Kidney. A demonstration of the medullary structure of a normal kidney with several glomeruli in their capsules surrounded by cut sections of convoluted tubules. The field also includes some collecting tubules and a diagonal section through at least one fairly large blood vessel. B, Kidney. A higher magnification of a glomerulus in its capsule. Characteristic cell lining is evident in the neighboring convoluted tubules.

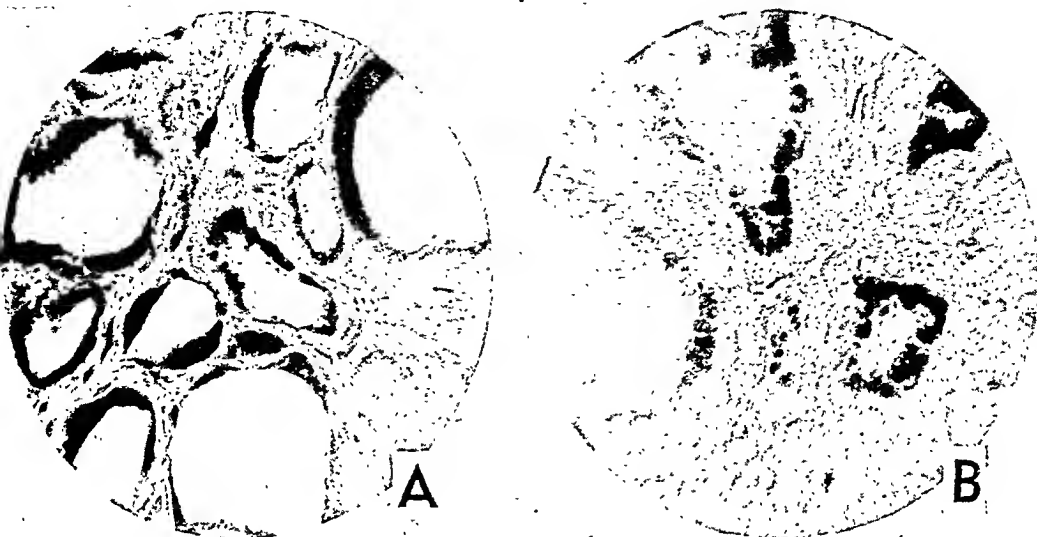


Fig. 4.—A, Normal thyroid. Section shows the typical arrangement of follicles, each well filled with homogeneous colloid substance and lined with a layer of flat epithelial cells. B, Toxic goiter. Hyperplasia is evidenced by the change of the epithelial cells to the characteristic columnar type. The entire tissue is much more cellular, but this example is not extreme because there is still a good deal of colloid substance and very little irregularity of the follicle outlines.

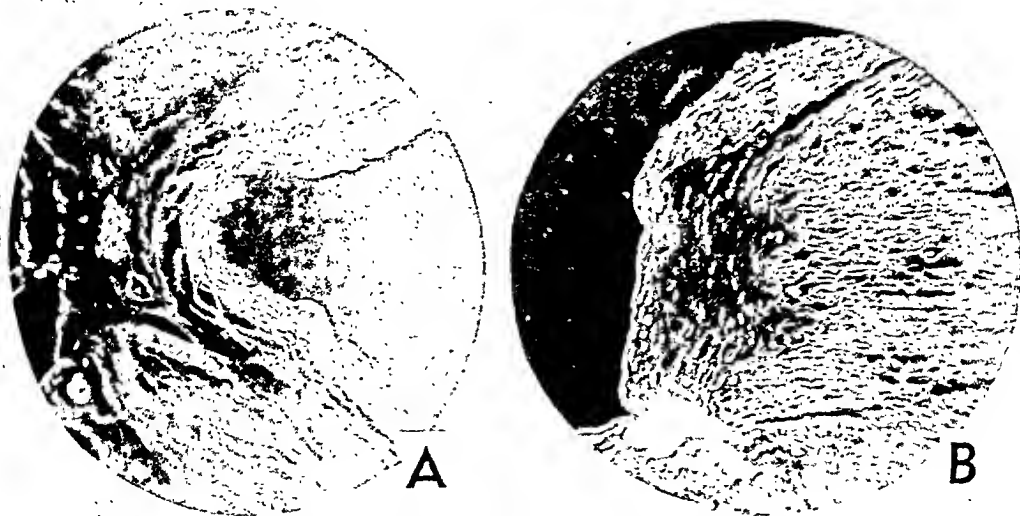


Fig. 5.—A, Subcutaneous vein. Section shows a portion of a fairly large vein embedded in loose connective tissue. The intima can be identified, but the media and the adventitia are indistinguishable. B, Thoracic aorta. This section is taken from the descending portion of the aortic arch at the point where the initial rupture of the intima and the media started a dissecting aneurysm. The elastic fibers of the media have retracted, allowing the cells of the intima to cover their ends.

added the claim that such a print will show a tissue section more nearly as it actually is—a thin slice of bloodless, white material outlined against a background of space, its delicate pattern of cell walls and nuclei sharply visible.

One improvised assembly of ordinary equipment is presented. (Fig. 1) with several examples of this new kind of photomicrography made as described. It is hoped that more pathologists and editors will approve of the positive projection print and give this simple method of photomicrography official sanction for more extensive use.

BOOK REVIEWS

Pathology of Tropical Diseases. By *J. E. Ash*, Colonel, M.C., U.S.A.; Director, Army Institute of Pathology, Army Medical Museum; and *Sophie Spitz*, M.D., C.S., A.U.S.; Pathologist, Army Institute of Pathology, Army Medical Museum. W. B. Saunders Company, Philadelphia. Price \$8.00. Cloth with 350 pages and 941 illustrations.

In no field of medicine is it more necessary for texts, monographs, and journal articles to carry good illustrations than in pathology. Hence there are two kinds of books in pathology, those with good illustrations and those with poor illustrations. The illustrations in this atlas are not good—they are superb. The authors have exercised good judgment in selection of subjects; Mr. Reeve and the staff of the Photographie Laboratory of the Army Institute of Pathology have outdone themselves in technical details; and the publishers have faithfully reproduced the originals.

Added value is given to the illustrations by the explanatory legends. The usual custom in texts is to append only a simple statement of the diagnosis. In this manual the legends are skillfully combined to form a brief exposition of the disease. For example, on pages 166, 167, and 168 there is a section on North American blastomycosis. The legends of the nine pictures read successively: "(1) Primary cutaneous blastomycosis causes papulopustular lesions with numerous small satellites surrounding the large discharging ulcers. The pitted scars are residuum of smallpox. (2) Healing leads to formation of crusts and atrophic scars in the center of the lesions while irregular extension of the process continues at the periphery thus accounting for the characteristic clusters. (3) Systemic blastomycosis involves the lungs more frequently than any organ, other than skin. The involvement is of diffuse miliary type, grossly indistinguishable from miliary tuberculosis. (4) Spleen is commonly involved and shows a few or many pale caseous foci. (5) Bone lesions may be osteolytic abscess cavities lined by pyogenic membrane. (6) A characteristic of primary cutaneous blastomycosis is the extreme pseudo-epitheliomatous hyperplasia with many ramifying epidermal extensions into dermis. The cavities thus formed are filled with purulent exudate and epithelial debris. The granulomata in the dermis are usually discrete. Numerous intra-epidermal and intradermal abscesses are present in this field. (7) Organisms (*Blastomyces dermatitidis*) are frequently found within giant cells of the granulomatous lesions of the dermis or within the intradermal abscesses. The fungi occur in tissue as single or budding thick-walled, double-contoured cells. (8) Distribution of pulmonary lesions is of a generalized, hematogenous type. Larger pneumonic areas may result from confluence of the miliary nodules. There is superficial resemblance to miliary tuberculosis. Note the necrotic center of many of these tubercles. (9) Granulomatous nodules show numerous multinucleated giant cells within a discrete collection of epithelioid cells and lymphocytes. Giant cells of the granulomata may contain the double contoured cell of *Blastomyces*. Endospores are always absent in contrast with *Coccidioides immitis*." This is a short but entirely adequate description of the salient features of the disease.

Each chapter, and there are twenty-two, has an introductory discussion of the diseases included, with a paragraph on definition, clinical features, and pathology. In the case of some, there is also a paragraph on epidemiology. In all, seventy-five disease entities are considered ranging from yellow fever to pinta, to heat exhaustion, to beriberi, to ainhum. There are a few selected references at the end of each chapter, and they are to recently published articles almost exclusively. For example, in Chapter 1, the dates are 1915, 1943, in press, 1944, 1944, 1944. Thus, only one is two years old and one is in the future.

Maps and diagrams are used to advantage to show geographic distribution and to clarify the stages in the life cycles of many of the metazoan parasites.

It is to be hoped that this book may be the first in a new viewpoint of American medical publishers. Frequently, one picture is worth more than hundreds of words. Medi-

cine should be an objective, documented science. In the nineteenth century many texts and atlases of pathology with excellent black and white and colored illustrations were published. Some authors cut their own lithograph plates. Perhaps a way can be found in the economically complex twentieth century to revive the practice of printing richly illustrated books. This atlas of Ash and Spitz proves that the authors, photographers, artists, and engravers are fully ready and that the publishers are willing.

ROBERT A. MOORE.

Diseases of the Breast. By *Charles F. Geschickter, M.A., M.D., Lt. Comdr., M.C., U.S.N.R.;* Director of the Francis P. Garvan Cancer Research Laboratory; Pathologist, St. Agnes Hospital, Baltimore, Md.; with a special section on treatment in collaboration with *Murray M. Copeland.* Second edition, J. B. Lippincott Company, Philadelphia, Pa., 1945. Price \$12.00. Cloth with 826 pages and 593 illustrations.

The second edition of this book presents a definite improvement over the first in many details. The fundamental treatment of the subject, however, has not been essentially altered.

The chief appeal of this monograph rests in its biological approach to the subject. This has been competently handled. The normal development of the breast is considered in relation to the ovarian and hypophyseal hormones and their effect upon normal growth and development. The estrogen-progesterone balance in the production of normal mammary structure is well considered. The anatomic and clinical disabilities that result from an escape from this normal balance has been used as the basis of the understanding of the cystic and hyperplastic changes so often encountered in the human breast. An effort is made to divide these lesions into several subgroups based upon their pathologic and clinical features as well as urinary assays and animal experimentation.

In these subgroups, certain minor criticisms might well be raised. For instance, one would question the consideration of mastodynia, or painful breast, as a pathologic entity. One would also wonder why the lesions of fibroadenoma and intracystic or intraductal papilloma are considered as neoplasms when they so often demonstrate their endocrine origin. The estrogen-progesterone studies that have been made on clinical lesions have been excellently portrayed. It is unfortunate that more of them are not available.

The statistical studies on the relationship between chronic cystic mastitis and cancer of the breast are well presented. While not an exhaustive review of the literature is offered, enough important papers are considered to make this a fair consideration of the subject. Bleeding from the nipple is considered separately. In view of the fact that in the author's series such a high percentage (seven) of patients with bleeding from the nipple subsequently developed cancer in that breast, one finds reason to question the conservative treatment that he has to offer for this lesion. One would also find reason to question the effect of hormonal therapy suggested in various places in the monograph on the basis of the evidence presented.

In the section related to malignant mammary tumors, those factors influencing the development and prognosis are well considered. The rather loose division into infiltrating adenocarcinoma and circumscribed adenocarcinoma probably is well worth while from a prognostic standpoint. The clarity of this classification is obscured somewhat by the inclusion of so many subgroups related chiefly to their microscopic appearance. Such detailed morphologic classification of cancer is usually more confusing than enlightening.

Once again an effort to prognosticate what will happen to a particular patient with cancer of the breast, based solely on the microscopic appearance of the lesion, remains unconvincing. This reviewer notes with pleasure that Paget's disease of the nipple is considered as primary cancer of the ducts.

The surgical and radiologic treatment is adequately handled. The format of the book is excellent, the type pleasing to the eye, most of the illustrations well reproduced, and typographic errors rare. Both the author and the publisher deserve praise for this monograph.

NATHAN A. WOMACK.

OBSERVATIONS ON CAPILLARY AND VENOUS BLOOD IN TRAUMATIC SHOCK

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NEW YORK, N. Y.

THE simplicity of technique and apparatus and the speed of results have given the recently described copper sulfate¹ method for determining hematocrits and plasma proteins a well-deserved popularity. This method was used routinely in our evacuation hospital as a guide in the treatment of the shock in war wounds and in the controlled fluid therapy in pre- and postoperative patients.

Cannon and associates² in World War I noted a disproportionately high erythrocyte count in capillary as compared with venous blood in cases of wound shock. Based on this, the authors of the copper sulfate method caution against using capillary blood specific gravity in cases of shock. The suggestion is made that differences in specific gravity between capillary and venous blood might be used to diagnose the shut down of peripheral circulation. This implication is also made by Scudder and Self.³

Using the copper sulfate method in the manner to be described, simultaneous capillary and venous whole blood and serum gravities were done on seventy-seven war casualties in varying degrees of secondary shock. The comparative results proved of no value in the diagnosis of shock; in many instances capillary and venous blood serum can be used interchangeably. This communication is based on the seventy-seven cases.

METHOD

All capillary and venous gravity determinations were done by one individual. The venous blood was drawn from the antecubital vein whenever accessible and transferred to a tube containing 1 mg. ammonium-potassium oxalate mixture per cubic centimeter of added blood; 3 c.c. were allowed to clot in a test tube for serum.

Capillary blood was obtained at the same time; whenever accessible, the ear lobe was used. The skin was cleansed, dried, and punctured deeply with a lancette. The blood was drawn into a pipette and dropped immediately into the copper sulfate solutions. Any dropper with a moderately fine point can be used. Approximately ten drops of capillary blood were then collected in capsules for serum. Small test tubes, as suggested by Kagan,⁴ were first used but were soon discarded for the capsules described by Lyon.⁵ Satisfactory receptacles were made using glass tubing with an inside diameter of 3 mm.

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The length of the entire capsule was approximately 7 cm., with a capillary end about 1 cm. in length, and the "blowhole" was about 4 cm. from the closed end. There is very little difficulty in filling this tube if it is shaken down between drops. The blood was allowed to clot, the capillary end broken, and the clot separated from the walls. The open end was sealed with adhesive tape, placed in a corked centrifuge tube, and spun down.

For measuring specific gravities of whole blood, solutions graded at intervals of 0.004 were found sufficiently accurate. In the plasma range, solutions graded at intervals of 0.002 were used. In comparing whole blood gravities, 0.0005 was subtracted from the venous blood value to correct for the added anticoagulant. This was also subtracted from the venous plasma.

The procedure employed for the formol-gel test was essentially comparable to that used by most others. Two drops of 36 per cent formalin were added to 1 c.c. of serum, and one drop of formalin was added to 1 c.c. of plasma. The tubes were mixed by inversion, corked, and left at room temperature for twelve hours. Degrees of viscosity were estimated by tilting the tubes. Positive reactions were designated by the numbers 1 to 4, ranging from a slight change in viscosity to a solid gel.

RESULTS

Comparisons between capillary and venous blood in sixteen normal subjects are listed in Table I. In general, the comparative values for whole blood and hematocrits are in fairly close agreement. The first study on simultaneous capillary and venous serum protein was apparently made by Kagan, using the rate of fall of a drop of serum as a measure of its protein content. In fourteen normal adults and two children he found no difference more than 0.2 Gm. per 100 c.c. With our technique the capillary serum value is often

TABLE I. COMPARISON OF CAPILLARY AND VENOUS BLOOD IN NORMAL SUBJECTS

PATIENT	WHOLE BLOOD* SPECIFIC GRAVITY		SERUM PROTEINS (GM./100 C.C.)		PLASMA PROTEINS* (GM./100 C.C.)	CALCULATED HEMATOCRIT† (PER CENT)	
	VENOUS	CAP.	VENOUS	CAP.		VENOUS	CAP.
1	1.0605	1.060	7.0	6.8	7.2	47	47
2	1.0595	1.059	6.2	6.3	6.3	48	47
3	1.0575	1.058	6.2	6.5	6.3	45	45
4	1.059	1.060	6.3	6.6	6.5	47	48
5	1.0585	1.061	6.2	6.2	6.7	47	50
6	1.059	1.060	7.3	7.3	7.3	45	46
7	1.060	1.060	6.2	6.2	6.7	49	49
8	1.061	1.062	6.8	7.2	7.3	49	49
9	1.0595	1.059	6.5	7.2‡	7.2	47	45
10	1.0595	1.059	7.2	7.1	7.3	46	45
11	1.058	1.058	6.7	6.8	7.0	45	45
12	1.0565	1.056	6.5	6.7	6.7	43	42
13	1.0565	1.057	5.8	6.0	6.3	45	45
14	1.0585	1.058	6.3	6.7	6.5	46	45
15	1.0625	1.066	6.6	7.8§	7.0	51	54
16	1.0575	1.057	6.0	6.2	6.0	46	45

*0.0005 has been subtracted for the anticoagulant.

†In calculating the hematocrit from the line chart, the serum protein value was used.

‡Serum leaked out while centrifuging.

§Great deal of squeezing necessary; serum hemolyzed.

more than 0.2 Gm. higher. This might be explained by the difference in the manner of blood collection. Lowry and Hunter⁶ have recently compared serum specific gravity of venous blood with finger and ear blood and have found no significant difference in normal individuals.

Abnormally high capillary serum protein values will be obtained if the capillary tube is not sealed and the centrifuge tube not tightly corked during centrifugation. Excessive squeezing with hemolysis of the capillary blood usually results in high protein values. To measure the effect of squeezing the ear or finger on the capillary whole blood specific gravity, the weights of the first drops of blood obtained after puncturing were compared with the weight of drops obtained after the capsule had been filled. The average fall in specific gravity in the sixteen normal subjects was 0.002.

Examples of each type of injury causing traumatic shock are recorded and grouped in Table II. These include extremity wounds with extensive soft tissue laceration, extremity wounds with hemorrhage or fracture, chest wounds with hemothorax, abdominal wounds, combined abdominal and chest wounds, crush injuries, burns, and head wounds. The findings in the remainder of the seventy-seven cases studied are, for the purposes of this report, essentially the same. There is no consistent or significant difference between capillary and venous whole blood gravity. Any discrepancy fits into the normal range and can usually be accounted for by the error in the method.

The two cases of generalized edema included in Table II were studied to obtain the effect of edema on the capillary blood gravity. It is apparent from these as well as from the traumatic cases with generalized edema due to hypoproteinemia that the effect on the capillary values is not above the error in the method.

In fifty battle casualties the effect of squeezing on the capillary blood gravity was, in general, the same as in the normal subjects. The average decrease was 0.002 in drops collected before and after filling the capillary capsule, irrespective of the degree of shock or the presence of edema.

It was fully realized that the total serum protein did not indicate the proportion of albumin and globulin. Thus a hypoproteinemia due to a low albumin fraction would be concealed by a rising globulin concentration, the total protein being normal. There is no accepted simple rapid quantitative test for determination of albumin or globulin separately. The only alternative was to use a semiquantitative test for hyperglobulinemia. The serum formol-gel reaction is not strictly correlative with the serum globulin, yet it has been found to be positive in varying degrees in sera containing more than 3.5 Gm. globulin per 100 c.c.⁷⁻¹⁰ In over 250 determinations on seventy-seven severely wounded soldiers, many of whom were checked from eight to ten days after injury, the reaction was positive (1 plus) on only three occasions.

The normal difference between the plasma and serum protein, which is apparently due mainly to fibrinogen, averages 0.4 Gm. per 100 c.c. of plasma (Table I). The danger of using this factor to interchange plasma and serum

TABLE II. CAPILLARY AND VEINOS

PA- TIENT	DATE OF IN- JURY	TIME OF IN- JURY	TYPE OF INJURY	TIME BLOOD COLLECTED	B/P	PULSE	GENERAL CONDI- TION AND COM- MENT	CONDITION OF SKIN AND EDEMA
R. H.	10/8	2 P.M.	Traumatic amputation and extensive muscle evulsion, right lower arm and shrapnel wound right thigh	10/8 6 P.M.	60/40	140	Poor	Pale, cool
				10/9 9 A.M.	120/60	106	Fair	Warm, pink
				10/10 9:30 A.M.	120/78	108	Fair	Warm, pink
II. H.	10/9	8 A.M.	Perforated wound left ankle, thigh, calf, but- tock, and right foot, with muscle evulsion	10/10 10 A.M.	95/45	160	Poor	Warm
				10/11 9:30 A.M.	118/70	160	Poor; deliri- ous	Warm, moist
				10/12 10 A.M.	112/70	160	Poor; deliri- ous	Warm
II. C.	12/1	8 P.M.	Traumatic amputation left foot, with compound comminuted fracture left leg and penetrating wound right leg and thigh	12/5 11 A.M.	88/40	140	Poor	Warm, pink
				12/6 9 A.M.	80/40	120	Poor	Warm, pink
C. L.	1/1	6 A.M.	Penetrating wound leg and thigh, with compound comminuted fracture femur; lacerated penis and scrotum; penetrat- ing wound arm and right thigh	1/1 1 P.M.	42/35	124	Bad	Pale, cool
				1/2 10 A.M.	75/45	132	Poor	Pinker, warm
Y. B.	1/2	8 P.M.	Land mine; compound comminuted fracture both ankles and destruc- tion right foot	1/3 3 P.M.	70/50	160	Bad; irra- tional	Pale, moist
G. W.	10/12	1 P.M.	Gunshot wound left ankle, thigh, and penis; super- ficial wound back	10/12 6 P.M.	80/60	110	Poor	Pale, warm
				10/13 9:30 A.M.	105/70	88	Good	Warm, pink
J. H.	11/21	--	Shrapnel wounds both legs with compound comminuted fracture tibia; exposed three days	11/25 11:45 A.M.	50/30	100	Bad; uncon- scious; died 12/25, 1:30 P.M.	Pale
F. W.	11/30	3 A.M.	Land mine; multiple wounds arm, ankles, and forearm	11/30 11 A.M.	140/70	160	Bad	Warm, pink
H. V.	12/4	12 M.	Gunshot wounds both thighs with compound fracture left femur	12/4 7 P.M.	0/0	120	Bad; uncon- scious; died 12/4 11 P.M.	Pink, cool

*The United States blood contained 500 c.c. of anticoagulant solution to 500 c.c. of blood.

D STUDIES IN TRAUMATIC WOUNDS

PREVIOUS TREATMENT AND FLUID THERAPY	WHOLE BLOOD SPECIFIC GRAVITY		SERUM PROTEIN (GM. PER CENT)		PLASMA PROTEIN (GM. PER CENT)	PLASMA FORMOL- GEL	CALCULATED HEMATOCRIT (PER CENT)	
	VENOUS	CAP.	VENOUS	CAP.			VENOUS	CAP.
	1.0505	1.053	4.8	5.8	5.5	0	39	40
amputation at right upper limb 10/8, 11 P.M.; 2,000 c.c. United States* blood and 1,000 c.c. plasma I.V. therapy	1.0405	1.0405	5.5	5.3	5.6	0	23	24
debridement some wounds, 10/10, 2 A.M.; 500 c.c. plasma and 2,000 c.c. United States blood	1.0415	1.041	5.5	5.5	5.6	0	25	24
debridement other wounds, 10/10 9 P.M.; 1,000 c.c. United States blood c.c. plasma	1.0465	1.046	4.8	4.8	5.3	2	34	33
	1.0445	1.044	5.0	5.1	5.5	4	30	29
1 c.c. plasma 12/4, 12 M.; amputation left thigh and débridement wounds 12/5, 6 A.M.; 100 c.c. plasma, and 100 c.c. United States blood I.V. therapy	1.0435	1.0445	5.1	5.5	5.3	0	29	29
c.c. plasma, 1/1, 30 A.M.	1.0405	1.040	4.9	4.8	5.3	0	25	25
	1.0405	1.041	5.1	5.3	6.0	Opales. 0 Opales.	24	25
1 c.c. blood and 900 c.c. plasma; amputa- tion thigh and testicle, 12/2, 1:30 A.M.	1.0395	1.038	5.1	5.3	5.6	0	23	21
1 c.c. blood and 1,500 c.c. plasma	1.048	1.049	5.8	6.0	6.0	0	33	34
I.V. therapy	1.051 R.B.C., 3.4	1.052 R.B.C., 3.55	5.0	5.5	5.3	0	39	39
debridement wounds, 12/12, 10 P.M.; 1,000 c.c. blood	1.048	1.046	5.1	5.0	5.3	0	35	32
c.c. plasma and 2,000 c.c. United States blood	1.0435	1.043	5.6	5.8	6.2	0 Opales.	27	26
c.c. plasma at 5 A.M.	1.0445	1.044	6.0	6.0	6.5	0 Opales.	27	27
c.c. plasma and 100 c.c. United States blood	1.0395	1.039	5.3	5.4	5.6	0	22	22
Opalescent.								

PATIENT	DATE OF INJURY	TIME OF INJURY	TYPE OF INJURY	TIME BLOOD COLLECTED	B/P	PULSE	GENERAL CONDITION AND COMMENT	CONDITION OF SKIN AND EDEMA
T. J.	12/4	8 A.M.	Shrapnel wound left hand	12/5 7 P.M.	50/0	120	Bad; congestive failure	Warm, pink with ankle edema
				12/6 9 A.M.	100/64	100	Poor	Warm, pink
W. H.	4/11	8 P.M.	Gunshot wound buttock with compound fracture ileum	4/12 4:30 P.M.	60/0	120	Bad	Pale
R. E.	10/8	10 A.M.	Shrapnel wound upper arm with lacerated brachial artery and vein	10/8 3:30 P.M.	94/58	128	Poor	Pale, cool
				10/9 9 A.M.	128/82	116	Good	Warm, pink
C. R.	11/25	7 P.M.	Shrapnel wounds of both thighs and scrotum, with lacerated femoral vein	11/26 3 P.M.	90/55	124	Poor	Pale; no edema
				11/26 7 P.M.	75/40	128	Poor	Pale; no edema
				11/27 9:30 A.M.	100/65	104	Fair	Warm
				11/28 11:30 A.M.	100/60	100	Fair	Warm
M. F.	4/12	12 M.	Gunshot wound thigh with lacerated femoral vein and scrotum	4/12 4:30 P.M.	70/40	80	Bad	Pale
				4/13 10 A.M.	105/60	72	Poor	Pinker
K. J.	11/8	4 A.M.	Shell wound left chest with hemothorax	11/10 9 A.M.	150/100	120	Fair	Warm
M. J.	12/8	--	Shell wound chest and multiple wounds knee with compound comminuted fracture of femur	11/11 8:30 A.M.	130/90	128	Fair	Warm
				12/16 12 M.	120/80	120	Poor	Pale
				12/17 3 P.M.	140/88	88	Fair	Pinker
H. J.	12/31	10:30 A.M.	Shrapnel wound abdomen with lacerated jejunum	12/31 2:30 P.M.	80/65	84	Bad	Warm, moist, pink
D. W.	11/30	3 A.M.	Land mine; multiple perforations of intestine; traumatic amputation right foot	12/31 9 P.M.	135/80	88	Fair	Warm, dry, pink
				11/30 11 A.M.	100/40	126	Bad	Warm, pale
				12/2 2 P.M.	135/70	108	Poor	Moist, pink; died 12/11

CONT'D

PREVIOUS TREATMENT AND FLUID THERAPY	WHOLE BLOOD SPECIFIC GRAVITY		SERUM PROTEIN (GM. PER CENT)		PLASMA PROTEIN (GM. PER CENT)	PLASMA FORMOL- GEL	CALCULATED HEMATOCRIT (PER CENT)	
	VENOUS	CAP.	VENOUS	CAP.			VENOUS	CAP.
amputation two fingers, 12/4, 6 P.M.	1.0495	1.050 Finger 1.052 Ear	5.1	5.1	5.6	0	37	37
0 c.c. plasma; three ampules of digitalis	1.0515	1.052 Finger 1.053 Ear	6.2	6.3	6.5	0 Opales.	37	37
0 I.V. therapy	1.0515	1.051	6.2	6.5	6.7	0 Opales.	37	35
0 c.c. plasma, 10/8, 11 P.M.	1.0485	1.047	5.6	5.5	5.6	0	34	38
debridement of wounds and ligation brachial artery and vein; 1,000 c.c. United States blood	1.0445	1.045	4.8	4.8	5.0	0	31	32
000 c.c. blood and 1,000 c.c. glucose and saline; debridement wounds, and ligation femoral vein, 11/20, 9 A.M.	1.043	1.042	3.8	3.8	4.3	0	32	31
0 I.V. therapy	1.043	1.042 Finger and ear	3.8	3.9	4.1	0	32	30
000 c.c. blood and 1,000 c.c. plasma	1.0455	1.0455	5.1	5.4	5.6	0 Opales.	22	21
amputation right knee, 11/27	1.0445	1.044	5.1	5.2	5.6	4	20	29
000 c.c. of plasma, 12/4, 2 P.M.	1.0415	1.039	5.5	5.5	5.6	0	25	22
000 c.c. United States blood, 500 c.c. plasma, and 1,000 c.c. glucose and saline	1.0385	1.038	5.1	5.1	5.6	0	22	21
amputation of 150 c.c. blood, 11/8; 1,000 c.c. United States blood, and 1,000 c.c. glucose and saline	1.058	1.056	5.8	6.0	6.7	4	47	44
000 c.c. glucose and sa- line; drinking freely	1.056	1.055	5.5	5.3	6.3	4	45	44
varicosotomy, 12/8, and debridement of leg	1.0495	1.050	5.3	5.6	6.2	4	36	26
amputation at thigh, 12/16; 1,000 c.c. blood, 1,000 c.c. glucose and saline, and 500 c.c. plasma	1.0505	1.050	4.8	5.1	5.6	4	39	38
0 I.V. therapy	1.061	1.061	6.2	6.8	6.4	0	50	49
0 c.c. plasma and 1,000 c.c. United States blood	1.061	1.060	6.2	6.8	7.2	0	50	47
0 c.c. plasma, 5 A.M.	1.045	1.044	5.8	6.0 Hemolyzed	6.3	0	29	27
amputation at hip; peri- osteitis; 4,000 c.c. glu- cose and saline, 1,000 c.c. blood, and 250 c.c. plasma, 12/1	1.0515	1.0485	5.3	5.3	6.0	4	39	35

PA-TIENT	DATE OF IN-JURY	TIME OF IN-JURY	TYPE OF INJURY	TIME BLOOD COLLECTED	B/P	PULSE	GENERAL CONDI-TION AND COM-MENT	CONDITION OF SKIN AND EDEMA
D. A.	12/26	--	Shell wound chest into ab- domen through spinal cord	11/28 11:30 A.M.	95/65	132	Poor	Warm, pink
				11/28 7 P.M.	90/50	132	Poor; died 12/4	Moist; jaundiced
C. R.	12/29	2 P.M.	Shell wound fracturing kidney, through liver, into lung with pneumo- hemothorax	12/30 10 A.M.	125/100	108	Poor	Pale, moist, warm
				12/31 10 A.M.	145/85	140	Poor	Edema of ankles
R.B.	10/21	9 A.M.	Vehicle overturned; crush injury chest with frac- tured humerus	10/21 10 A.M.	80/60	140 (Irreg.)	Poor	Warm, pink
				10/21 2 P.M.	30/20	140	Poor	Warm, pink; died, 3 P.M.
M. W.	11/7	3 P.M.	Crushed between wall and truck; multiple rib fracture	11/7 6:30 P.M.	70/40	140	Poor	Pale, cold
				11/8 8:30 A.M.	100/60	120	Fair	Warm
M. H.	10/11	8 A.M.	Second-degree burns of 62 per cent body sur- face	10/12 9:30 P.M.	100/80	124	Poor	Edematous
				10/13 10 A.M.	118/80	128	Poor	Edematous
				10/13 8:30 P.M.	128/60	138	Poor	Edematous
K. E.	4/16	4 A.M.	Second-degree burns, legs, hand, face; 45 per cent body surface	4/16 10:30 A.M.	100/80	112	Bad	Little leg edema
				4/17 9 A.M.	90/70	120	Poor	Little leg edema
				4/18 9:30 A.M.	90/60	124	Poor	Moderate leg edema
M. S.	4/16	4 A.M.	Second-degree burns face, wrist, thigh, and but- tock; 30 per cent body surface	4/16 11 A.M.	125/85	64	Poor	-----
				4/17 9 A.M.	100/65	60	Fair	No edema
H. N.	11/24	7:30 A.M.	Compound comminuted fracture frontal bone with evisceration and lacerated brain with extensive hemorrhage	11/24 10 A.M.	85/50	80	Bad; uncon- scious	Pale
D. R.			Nephrosis	5/9	170/110			Edema face, hands, and legs, and ascites
				5/13				Edema face, hands and legs with ascites
W. R.			German prisoner for eight months; nutritional de- ficiency					Edema face, hands and legs with ascites

PREVIOUS TREATMENT AND FLUID THERAPY	WHOLE BLOOD SPECIFIC GRAVITY		SERUM PROTEIN (GM. PER CENT)		PLASMA PROTEIN (GM. PER CENT)	PLASMA FORMOL- GEL	CALCULATED HEMATOCRIT (PER CENT)	
	VENOUS	CAP.	VENOUS	CAP.			VENOUS	CAP.
iriation of chest, 1/26; 1,000 c.c. blood, 1/27; splenectomy, 1/28 I.V. therapy	1.0545	1.054	5.5	5.8	6.3	1	43	41
oracotomy and laparot- omy, 12/30, 2 A.M., 1,000 c.c. blood at oper- ation 500 c.c. glucose and saline I.V. therapy	1.0575	1.0575	6.0	6.1	6.5	4	46	45
	1.055	1.053	5.5	5.8	5.7	0	43	40
	1.060	1.061	5.3	5.8	6.0	1	50	51
	1.0565	1.0535	5.4	5.6	5.3	0	46	41
0 c.c. plasma and 500 c.c. blood; autopsy showed hematoma of chest and back muscles 0 c.c. plasma	1.048	1.0485	5.1	5.1	5.4	0	34	35
	1.0515	1.051	5.5	5.5	5.6	0	38	38
0 c.c. plasma and 1,000 c.c. United States blood 500 c.c. plasma; local débridement	1.050	1.051	5.1	5.1	5.3	0	37	38
	1.0645	1.064 Toe	--	6.2 Squeezed hard	5.6	0 Opales.	--	54
500 c.c. plasma	1.0605	1.061 Toe	5.6	5.8	6.2	4	50	51
500 c.c. plasma	1.0555	1.053 Toe	5.5	5.6	6.5	4	44	41
500 c.c. plasma	1.0645	1.064	6.2	7.2	7.0	0	55	52
			Centrifuge leaked					
500 c.c. plasma and 1,000 c.c. 5 per cent glucose; local débride- ment	1.0625	1.064	5.8	6.2	6.0	0 Opales.	53	54
500 c.c. plasma; drink- ing freely	1.0605	1.059	4.8	5.1	5.7	4	52	49
0 c.c. plasma	1.0555	1.059 Ear	6.5	7.9	7.0	0	42	44
			Excessive squeezing					
al débridement; drinking freely	1.0535	1.054	6.2	6.6	6.7	0	40	39
0 c.c. plasma at 8 A.M.	1.0415	1.041 Finger	4.6	4.6	5.1	0 Opales.	27	27
	1.0465	1.045 Ear 1.045 Finger 1.046 Toe	4.5	4.4	5.0	0	34	33
	1.0475	1.046	4.4	4.4	5.0	0	36	34
	1.0425	1.040 Ear 1.042 Finger	2.7	2.7	2.9	0	34	31

TABLE III. RELATIONSHIP OF FIBRINOGEN, GRAVITY-CALCULATED FIBRINOGEN, AND THE FORMOL-GEL TEST

PATIENT	FIBRINOGEN		FORMOL-GEL		SERUM GLOBULIN (GM./100 C.C.)
	GRAVITY CALCULATED* (GM./100 C.C.)	TYROSINE (GM./100 C.C.)			
			PLASMA	SERUM	
1	1.0	0.486	4	0	2.86
2	0.9	0.565	4	0	
3	0.9	0.470	1	0	1.00
4	0.8	0.285	0	Opales.	
5	0.8	0.532	3	0	
6	0.8	0.476	4	0	
7	0.8	0.476	1	Opales.	
8	0.8	0.600	4	0	
9	0.8	0.540	4	0	
10	0.8	0.500	4	0	1.40
11	0.7	0.361	0	0	2.50
12	0.7	0.361	0	0	2.11
13	0.7	0.322	3	0	
14	0.7	0.470	0	0	2.73
15	0.7	0.460	Opales.		
16	0.5	0.470	4	0	1.00
17	0.5	0.402	2	0	
18	0.5	0.580	0	0	
19	0.5	0.560	Opales.	Opales.	
20	0.5	0.480	4	0	
21	0.5	0.580	1	0	
22	0.5	0.430	4	0	2.44
23	0.5	0.400	1	0	1.66
24	0.5	0.290	2	0	3.40
25	0.5	0.302	Opales.		
26	0.5	0.340	0	0	1.30
27	0.4	0.386	0	0	1.75
28	0.4	0.340	0	0	1.80
29	0.4	0.300	Opales.		
30	0.3	0.393	0	0	
31	0.2	0.328	4	0	2.20
32	0.2	0.330	0	0	
33	0.2	0.389	1	0	1.25
34	0.1	0.344	0	0	2.10
35	0.0	0.340	Opales.		
			0	0	2.54
			4	0	

*0.0005 was subtracted from the plasma gravity for the anticoagulant.

values, or of inconsistently using the serum value and then the plasma, is evident from the data in Table II. The error is particularly large in those conditions in which fibrinogen production is stimulated;¹¹ in this series these include burns, extremity wounds with destruction of large muscle masses, penetrating chest wounds with hemorrhage and effusion, and abdominal wounds with peritonitis or hemoperitoneum.

In Table III are data comparing fibrinogen* values with plasma-serum gravity differences. In general, the greater the gravity difference, the higher the fibrinogen value, but no linear relation exists. A closer association exists

*Captain A. Saifer, Sanitary Corps, Seventh Medical Laboratory, performed the fibrinogen and globulin^{12, 13} determinations.

between the plasma formol-gel reaction and the fibrinogen level. With four exceptions (Cases 13, 30, 32, and 35) a positive formol-gel is associated with a fibrinogen of 400 mg. per cent or higher. All fibrinogen levels between 400 and 500 mg. per cent are associated with a positive gel of some degree or a definite opacity. Plasma with fibrinogen between 500 and 600 mg. per cent invariably gives a strong reaction.

The serum globulin, when present in normal amount, does not appear to have a significant effect on the formation of the plasma gel. Inasmuch as serum containing more than 4 Gm. per cent globulin forms a gel on the addition of formaldehyde, it is very probable that a certain fraction of the serum globulin, when present in excessive amounts, enters into the formation of the plasma gel. Wise and Gutman,⁸ in a study of the serum formol-gel reaction as a test for hyperglobulinemia, warn against the "false positive reactions" occurring in plasma while the corresponding sera are negative. They declare that even moderately increased fibrinogen will cause the plasma reaction to become positive. From our findings it is evident that a close parallelism exists between the amount of fibrinogen and the intensity of the plasma reaction, when the serum globulin is within normal limits.

DISCUSSION

The inability to corroborate Cannon's findings of increased capillary erythrocyte concentration with specific gravity measurements requires examination for the differences in subjects and technique. It is true that shock is treated earlier and more vigorously with plasma and blood in World War II than in World War I. Yet we have studied a sufficient number of patients who had been given no previous infusions, as well as a larger number that were seen in severe shock after having received a minimal amount of plasma and blood. It is unlikely that our consistently negative findings can be due to the more intensive therapy in World War II.

The amount of blood taken from the ear or finger assumes importance if we admit, as Cannon suggests, that in shock the blood corpuscles accumulate in the capillaries of the skin. At best, blood procured by lancet is an admixture of capillary, venous, and arterial blood. The more blood we express, the more it is mixed with venous and arterial blood. Although Cannon does not describe the technique in detail, it may be assumed that the first or second drop was used for the erythrocyte determination. Moreover, a smaller drop is needed for a red cell count than for our gravity measurement. With our method the capillary gravity range could not be accurately anticipated, and from one to three drops of capillary blood were expressed before the correct specific gravity was obtained. The actual effect of this difference in technique could be determined by either using a falling drop method which estimates the rate of fall of a single drop of blood, or by doing simultaneous erythrocyte, hemoglobin, and specific gravity determinations on the capillary blood. Lack of time and facilities prevented us from doing this.

There are several facts which are not easily explained by technical differences. First, Cannon ran hematocrit and hemoglobin determinations and erythrocyte counts in seven cases of shock and found the results to be comparable. Second, in the last stage of our study we determined the venous blood gravity first and then dropped the first drop of capillary blood in this range. Four patients in shock were examined in this manner; one exhibited a capillary gravity 0.004, another 0.003 higher than that of the venous blood (corresponding to a hematocrit 5 volumes per cent and 4 volumes per cent higher, respectively), and the other two showed no significant change. Third, the squeezing associated with filling the capillary capsule caused essentially the same drop in capillary blood specific gravity in those patients in shock as it did in normal subjects.

SUMMARY

1. Simultaneous comparisons of capillary and venous blood gravity in seventy-seven severely wounded soldiers showed no tendency of the capillary blood to be more concentrated than the venous. The gravity difference between capillary and venous blood proved of no value in the diagnosis of the severity of shock.

2. With due attention to the sources of error, capillary blood may be used in place of venous blood for determining hematocrits and serum protein. The effect of shock or edema on capillary values is not greater than the error in the method.

3. As calculated from specific gravities, the proteins of serum are from 0.5 to 1.0 Gm. per 100 c.c. lower than the plasma proteins in those conditions in which fibrinogen production has been stimulated. These conditions include burns, wounds with abundant muscle destruction, chest wounds with hemothorax and effusion, and abdominal wounds with peritonitis and hemoperitoneum.

4. In over 250 determinations on wounded soldiers studied up to ten days after injury, the serum formol-gel reaction was faintly positive on only three occasions. There is a close correlation between the fibrinogen level and the plasma formol-gel reaction. The latter does not appear to be affected by serum globulin that is within the normal range.

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STUDIES IN COMPLEMENT FIXATION

II. PRESERVATION OF SHEEP'S BLOOD IN CITRATE DEXTROSE MIXTURES (MODIFIED ALSEVER'S SOLUTION) FOR USE IN THE COMPLEMENT FIXATION REACTION

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THE increasing use of preserved human blood for transfusion purposes has stimulated investigations designed to establish ideal blood-preserving solutions.¹⁻⁸ The best of these have employed varying isosmotic concentrations of dextrose, citrate, and sodium chloride at slightly differing hydrogen-ion concentrations. The blood is stored in the various solutions in final concentrations ranging from 20 to 50 per cent, the dilution factor itself apparently influencing the efficiency of preservation. Kendrick and associates⁹ recently found modified Alsever's solution⁶ as acceptable as any tested and, employing this solution, developed a practical method of supplying preserved blood to the European Theater of Operations.

A preservation technique for maintaining uniformity in the properties of sheep's erythrocytes would be a great laboratory convenience. Most laboratories at present prepare suspensions of cells from defibrinated blood or blood collected into sodium citrate solution with or without the formalin advocated by Kohner¹⁰; cells so preserved have been stated to remain satisfactory for use over short periods. It would be of great advantage to employ preserving fluids which would maintain the properties of sheep's blood over long periods and during practical methods of transportation. Quantitative studies in this laboratory indicate that the aseptic collection of sheep's blood in modified Alsever's solution at ordinary temperatures and subsequent refrigeration permits the preservation of the blood for at least ten weeks (the duration of the experiment) without development of appreciable hemolysis or change in susceptibility to lysis by guinea pig complement and rabbit antioeceptor.

EXPERIMENTAL

Glass vaccine vials, calibrated at 30 ml., were fitted with rubber stoppers and evacuated. Using aseptic precautions, a series of eight vials were prepared to contain, respectively, 6, 9, 12, 15, 18, 21, 24, and 27 ml. of sterile modified Alsever's solution. Five milliliters of sterile 4 per cent sodium citrate were added to a ninth vial. Each vial was then aseptically filled to the 30 ml. mark with sheep's blood obtained at a single bleeding with the aid of a donor set of

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*Each 100 ml. of Alsever's solution contains: dextrose, 2.05 Gm.; sodium citrate, 0.8 Gm.; and sodium chloride, 0.42 Gm. The original Alsever formula has been recently modified by the addition of 0.055 Gm. of citric acid per 100 ml. The finally sterilized solution has a pH of 6.1 and shows very slight or no evidence of caramelization.

the type described by Elliott and Nessett.¹¹ The resulting suspensions, varying in blood concentration from 10 to 80 per cent, were stored at from 3 to 6° C. for the duration of the experiment and were mixed only before each removal of samples for analysis. At intervals of two, six, ten, seventeen, thirty-four, fifty-three, and seventy-four days after bleeding, the blood suspensions were mixed thoroughly by inversion and appropriate quantities removed aseptically. Portions containing 0.25 ml. of each mixed sample were added to a quantity of distilled water calculated to yield a 1:125 final dilution of the whole blood content of the sample, and the optical density of the resulting lysate was determined on the Coleman Junior Spectrophotometer* at 580 m μ . A portion of the supernatant fluid of the sample, obtained after centrifugation, was similarly diluted and the optical density determined. The optical density of the diluted supernatant divided by the optical density of the diluted whole blood lysate \times 100 was taken as the percentage of spontaneous hemolysis which had occurred during storage. Samples of each mixture were then washed with four changes of M/200 phosphate buffered saline, pH 7.3, and the volume of packed cells read in calibrated centrifuge tubes after a final centrifugation at 2,000 r.p.m. for ten minutes. The packed cells from each sample were brought to 2 per cent concentration by diluting to 50 volumes with buffered saline, and the optical density of a lysed 1:5 dilution of each 2 per cent suspension in distilled water was determined at 580 m μ .

A portion of the 2 per cent suspension made from the 50 per cent blood mixture was sensitized with an equal volume of the optimal rabbit amboceptor dilution,¹² and the quantity of guinea pig complement required for 50 per cent hemolysis was determined by spectrophotometric titration.¹³ Then portions of the other 2 per cent suspensions were sensitized similarly with rabbit amboceptor, and the percentage of hemolysis obtained with the above 50 per cent unit of complement was determined for each suspension. The 50 per cent unit for each suspension then was calculated with the aid of predetermined volume factors,¹³ and the remainder of the 2 per cent suspensions was stored overnight at from 3 to 6° C. On the following day portions of the stored 2 per cent suspensions were sensitized, and the 50 per cent hemolytic units again were determined in the same fashion using the lot of complement employed for the previous day's tests. Finally, a portion of each stored 2 per cent suspension was centrifuged at 2,000 r.p.m. for ten minutes, and the optical density of the undiluted supernatant fluid was determined at 580 m μ . The percentage of cells hemolyzed during overnight storage of the 2 per cent suspension was determined from the following formula:

$$\text{Percentage of hemolysis} = \frac{\text{Optical density of undiluted supernatant}}{\text{Optical density of 2 per cent cells} \times 5} \times 100$$

RESULTS

The 80 per cent blood mixture, in which the final concentration of citrate was only 0.16 per cent, had clotted completely after six days, and the 70 per

*Model 6, manufactured by Coleman Electric Co., Maywood, Ill., and New York, N. Y.

TABLE I. QUANTITATIVE STUDIES WITH PRESERVED SHEEP'S BLOOD

I BLOOD MIXTURE	II DAYS AFTER BLEEDING	III SAMPLE (ML.)	IV PACKED CELLS (ML.)	V O.D.* OF MIXTURE 1:125	SUSPENSION		RESULTS WITH SUSPENSION A STORAGE OVERN	
					VI O.D. OF HEM- OLYZED 2% CELLS	VII 50% UNIT† (ML. X 10-3)	VIII 50% UNIT† (ML. X 10-3)	HEM DURING (PER
IV	2	3.0	0.62	.490	.514	1.93	2.0	
Alsever's (15 ml.)	6	3.0	0.62	.458	.523	1.80	1.87	
Blood (15 ml.)	10	3.0	0.70	.470	.485	1.63	1.59	
	17	3.0	0.68	.450	.510	1.81	1.55	
	34	3.0	0.55	.472	.512	1.78	1.86	
	53	3.0	0.66	.452	.495	1.63	1.57	
	74	3.0‡	0.62	.490	.474	1.54	1.61	
IX	2	2.0	0.70	.462	.497	1.95	2.08	
4 per cent citrate	6	2.0	0.75	.452	.483	1.78	1.80	
(5 ml.)	10	2.0	0.84	.450	.479	1.64	1.64	
Blood (25 ml.)	17	2.0	0.80	.472	.503	1.80	1.58	
	34	2.0	0.80	.470	.497	1.79	1.77	
	53	2.0	0.75	.460	.498	1.62	1.65‡	
	74	2.0§	0.70	.452	.456	1.55	1.69‡	1

*O.D., Optical density determined at 580 mμ.

†Complement unit.

‡1.8 per cent hemolysis in this sample.

§2.5 per cent hemolysis in this sample.

¶These represent the unit of complement required for the hemolysis of residual intact cell course of the titration the color due to spontaneous hemolysis of the 2 per cent suspension was by the use of a blank prepared from its supernatant fluid.

cent blood mixture showed a few small clots after several weeks. Clotting not occur in the remaining mixtures. In Table I are summarized the data obtained with the 50 per cent blood-Alsever's mixture (Suspension IV) and parallel data obtained with the citrated blood mixture (Suspension IX). Results obtained with the 20, 30, 40, and 60 per cent blood mixtures were as satisfactory as those recorded for the 50 per cent mixture. The yields of packed cells obtained and the optical densities of the original blood mixtures and of the 2 per cent suspensions prepared therefrom were quite constant, suggesting that there was no tendency toward irreversible change in cell size during the course of storage. Spontaneous hemolysis of 2.5 per cent was observed in the citrate blood mixture after seventy-four days and was only slightly greater than that observed with the 50 per cent Alsever's mixture. Daily variations in the absolute quantity of complement required for 50 per cent hemolysis of the 2 per cent suspensions from the given mixture are the expected variations obtained when different ampules of sublimated complement are employed. The 50 per cent unit required for Suspension IV was also in very close agreement with that required for Suspension IX. It is especially noteworthy that the 2 per cent suspensions after storage overnight remained quantitatively unchanged in their response to lysis by complement and amboceptor. This was equally true of the *unhemolyzed* cells of the citrate blood mixtures at fifty-three and seventy-four days. The use of these 2 per cent suspensions in complement fixation tests employing an overnight fixation period, however, is contraindicated by the occurrence of 5 and 11 per cent hemolysis in the stored 2 per cent suspensions.

Such a degree of hemolysis leads to errors in estimating the degree of inhibition of hemolysis by serum plus antigen in an overnight fixation test. Since many complement fixation procedures are most sensitive after eighteen hours of refrigeration of test mixtures, this diminishes the usefulness of cells preserved in citrate alone.

This laboratory has tentatively adopted the use of modified Alsever's solution for the preservation of sheep corpuscles while additional studies are in progress. One hundred fifty milliliters of sheep's blood is collected aseptically in a sterile vacuum bottle containing 150 ml. of modified Alsever's solution. After thorough mixing, the mixture is aseptically dispensed into six sterile 50 ml. evacuated vaccine vials and stored at refrigerator temperature. Quantities of blood mixture are aseptically withdrawn from these vials as needed. Maintenance of strict asepsis is facilitated by introducing a sterile cotton-filled airway into the rubber cap before withdrawing the desired quantity of the blood mixture into a sterile syringe. In our experience with several sheep, 15.0 ml. of such a mixture will yield sufficient packed cells for 200 ml. of a 2 per cent suspension. On one occasion, a vial of preserved blood was flown to the Caribbean area where it remained at room temperature for two weeks and was then flown back to this laboratory. Upon arrival here, the mixture showed no hemolysis, and a 2 per cent suspension prepared from it required the same complement unit for 50 per cent hemolysis as a 2 per cent suspension prepared at the same time from a vial of the same mixture which had been refrigerated at this laboratory throughout this period. After three days in the refrigerator, however, the blood mixture which had been at room temperature for several weeks underwent considerable hemolysis. This was apparently a delayed osmotic hemolysis of the type encountered with human blood by DeGowin and associates² in the use of a modified Rous-Turner preserving mixture. Under ordinary circumstances, however, sheep's blood collected in Alsever's solution at room or mild outdoor temperatures can be subsequently refrigerated without undergoing osmotic hemolysis. This is similar to the experience of Kendrick and co-workers⁹ in the use of modified Alsever's solution for the collection of human blood. The uniform behavior of cells preserved in this fashion is further attested by the fact that the slope of the curve of hemolysis with complement remains constant throughout the period of storage.¹³

Several blood-citrate mixtures were also prepared according to the method of Ashby.¹⁴ Since considerable hemolysis occurred fairly rapidly, both in these stored mixtures and in washed 2 per cent suspensions prepared from them, the recommended citrate mixture was considered inferior to Alsever's solution. Twenty-five milliliters of sheep's blood were also treated with 6 Gm. of Amberlite, as recommended by Steinberg.¹⁵ After only seven days of preservation, considerable hemolysis occurred following refrigeration overnight of 2 per cent suspensions prepared from this mixture. After eleven days of preservation there was rapid hemolysis of this blood, and packed washed cells were resuspended with difficulty. It should be noted, however, that the original cells and the residual unhemolyzed cells of the later mixtures showed quantitatively the

same susceptibility to lysis by complement as did cells preserved in citrate or citrate-dextrose mixtures.

Preliminary studies have also been made with a citric acid-sodium citrate-glucose mixture prepared as recommended by Loutit and associates.² Moderate clot formation occurred shortly after the mixture was made, probably due, as Loutit and associates suggest, to the relatively low concentration of citrate radical which required that greater care be taken with this solution to assure thorough mixing. In addition, 19 per cent hemolysis had occurred in the mixture after only forty-eight days of storage, an amount considerably greater than that encountered with Alsever's solution.

No attempt was made to assess the comparative value of 90 per cent blood mixtures containing final concentrations of 0.5 per cent sodium citrate and 2 per cent dextrose as recently recommended by Coffin.¹⁰ It should be noted that Coffin encountered considerable hemolysis in his mixture exposed to 85° F., which constitutes a disadvantage in its use. It is possible that these mixtures undergo isosmotic hemolysis of the type described by DeGowin and co-workers.³

DISCUSSION

The duration of preservation of sheep's blood for use in the complement fixation reaction is dependent upon the same factors which influence the preservation of human blood; these factors are: (1) strict asepsis during the withdrawal, storage, and daily removal of the blood sample, (2) adequacy of the preserving fluid and the use of optimal blood-preserving fluid ratios, and (3) storage under refrigeration.

The necessity of maintaining absolute asepsis in all stages of handling the sheep's blood cannot be overemphasized. Contamination of the stored mixture will ordinarily result in prompt and progressive hemolysis of the cells. The use of commercially available* sterilized and evacuated bottles containing the required amount of modified Alsever's solution greatly facilitates maintenance of absolute asepsis. An evacuated 300 ml. bottle containing 150 ml. of Alsever's solution provides a most convenient unit for the collection of 150 ml. of blood. Under these conditions it is not necessary to use a bleeding set equipped with a valve; a short length of rubber tubing equipped with a small screw clamp and fitted with a 16-gauge needle at one end and 17-gauge at the other will serve as a most satisfactory bleeding unit.

The present investigation has not attempted an exhaustive survey of preserving fluids for use in prolonged storage of sheep's blood. Modified Alsever's solution, used with an equal volume of blood as described, proved sufficiently satisfactory to meet the needs of the average serologic laboratory, and it has been adopted for that reason. Later studies may well define other preserving fluids capable of extending the time of storage of sheep's blood well beyond that described in the present instance.

It is of considerable interest that blood preserved in Alsever's solution will survive storage at room temperature for appreciable periods. This property

*Baxter Centri-Vac, manufactured by Baxter Laboratories, Inc., Glenview, Ill., and College Point, N. Y.

makes it possible to transport such blood mixtures for considerable distances without necessitating refrigeration during shipment. The limitations of room temperature shipment as a practical method of dispatching sheep's blood from a central distributing point are still under investigation.

SUMMARY

Sheep's blood, aseptically collected in equal volumes of modified Alsever's solution at ordinary temperatures, may be preserved under refrigeration for at least ten weeks without significant alteration in susceptibility to lysis by complement and amboceptor. Blood collected in this fashion has been transported and maintained at ordinary temperatures for at least two weeks with no change in these properties.

The preservation of sheep's blood in modified Alsever's solution as described may not yet be the best available method. It has been exclusively used in this laboratory, however, during the past ten months with complete satisfaction.

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HOMOLOGOUS AND HETEROLOGOUS STRAINS OF PLASMODIUM VIVAX; A CROSS-INOCULATION STUDY OF MÁLARIA STRAIN IMMUNITY

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PREVIOUS investigations of the immune response to vivax malaria have demonstrated that strains of *Plasmodium vivax* differing in geographic origin are dissimilar in their stimulation of the immune mechanism.¹ This difference between substrains of the same species of malaria is best illustrated by the technique of cross-inoculation, whereby a heterologous strain produces definite clinical activity following the reinoculation of a patient who had previously experienced clinical activity with another strain. Homologous strains are represented by minimal or absent clinical activity on reinoculation in the same individual. The demonstration of these characteristics of homologous and heterologous strain immunity on a quantitative basis has aided in solving the provocative problem of dealing with the partially immune response to malaria inoculations in the treatment of neurosyphilis.

Variations in the susceptibility of different individuals to therapeutic malaria have been adequately considered in a previous publication.² Because of apparent native immunity to vivax malaria, all Negroes, Puerto Ricans, Mexicans, and Mediterranean peoples are primarily inoculated with quartan malaria at this institution. Similarly, all white patients with definite histories of previous attacks of natural malaria are initially inoculated with a quartan strain. The real problem, therefore, arose in the group of other white individuals, without definite histories of malaria, who experienced partially immune responses following vivax inoculation.

Partial immunity is recognized by the characteristics of both the clinical course and parasitemia of the inoculated individual. In these cases, parasites usually increase in density to several hundred per cubic millimeter before there is any febrile activity. Usually the parasite count drops spontaneously and precipitously after from three to seven paroxysms, and the patient becomes completely afebrile. Partially immune patients are divided into two groups: (A) Those experiencing less than five paroxysms of fever to 103° F. or more and (B) those experiencing from five to eight such episodes. This segregation was made to conserve time in the completion of a therapeutic course. At first it seemed logical, even though a previous history of natural malaria was not elicited from the patient, to reinoculate all partially immune patients with quartan malaria

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for the completion of their therapy. Two objections to this procedure were evident. First, reinoculation with quartan malaria, particularly in an individual who has already showed a certain degree of malaria immunity, was followed by a prolonged incubation and prepatent period, often lasting four or more weeks. This, plus the longer clinical course of quartan malaria (paroxysms every third day), substantially increased the hospitalization and morbidity of the patient. Second, the prolonged latency of quartan infections, reportedly thirty years or more in some instances, may actually play a role in the production of accidental transfusion malaria. In a recent report² of twelve patients with transfusion malaria in Massachusetts, nine of the twelve were quartan cases, two were tertian (*vivax*), and the etiologic species of one was not stated. It is, of course, recognized that these latent quartan infections were probably produced by the mosquito inoculation of the natural disease; and, although it is presently believed that blood inoculation is not followed by such latency, the procedure must be considered somewhat hazardous in this respect until further information is available. For these reasons, heterologous strains of *vivax* malaria were first utilized for the reinfection of those white patients demonstrating partially immune responses (Group B) to the original *vivax* inoculation. Group A patients were routinely reinoculated with quartan malaria because of their relatively high degree of *vivax* immunity. If adequate clinical activity (a total of eight to twelve paroxysms) can be produced by the use of heterologous strains, the prolonged reinoculation incubation period would be eliminated and the problem of prolonged latency obviated by the shorter *vivax* infection. It was the purpose of this study, therefore, to demonstrate the characteristics of homologous and heterologous strain immunity by a controlled cross-inoculation investigation. An additional refinement in the techniques of therapeutic malaria may thus become available.

The principle of reinoculation of previously *vivax*-infected individuals with heterologous strains of *P. vivax* is not a new one. Boyd,¹ in a description of the criteria of immunity and susceptibility in naturally induced *vivax* malaria infections, in 1942, demonstrated the difference between homologous and heterologous *vivax* immunity with different strains endemic to the United States. Of a series of twenty-five patients reinoculated with a known homologous strain of *P. vivax*, seventeen experienced subclinical "takes" and eight short clinical attacks. On the other hand, reinoculation in a series of nineteen patients with heterologous strains of *P. vivax* produced subclinical infection only once and clinical activity of varying duration in the remaining eighteen patients. The primary inoculation strain in that study was *P. vivax* McCoy, indigenous to Southeastern United States. The heterologous strains were obtained from other sections of the country.

The treatment at this installation of soldiers from overseas theaters facilitated the recovery of strains of *P. vivax* from patients originally infected in several different Southwest Pacific islands. These so-called Southwest Pacific strains, because of their more distant geographic origin, were utilized in this cross-inoculation study against the *P. vivax* McCoy strain. The results may be

of increasing value in the management of neurosyphilis with therapeutic malaria in a population manifesting greater malaria immunity as a result of the prolonged residence of soldiers in hyperendemic areas.

MATERIAL AND METHOD

Origin of Strains.—Five strains of *P. vivax* from different geographic regions were used in this study.

Strain M: The McCoy strain, carried through successive mosquito-human transfers for the past fifteen years at the Florida State Hospital, Chattahoochee, Fla. This strain was originally recovered from a patient infected in the Florida-Alabama area and is the one currently used for the primary inoculation of susceptible white patients.

Strain A: The DuBosc strain, recovered during the eighth malaria recurrence from a patient who first contracted the disease on Guadalcanal Island in November, 1942. He had had no previous experience with malaria prior to that attack and had been on no other Southwest Pacific island.

Strain B: The Jenkins strain, recovered during the second malaria recurrence from a patient first infected in February, 1944, on New Guinea Island.

Strain C: The Fort strain, recovered from a patient who had been on both New Guinea and Bougainville Islands, with the first attack of malaria occurring in the latter area.

Strain D: The Siimes strain, recovered during the fourth malaria recurrence from a patient whose first clinical attack occurred in December, 1942, on New Guinea Island.

Technique of Cross-Inoculation.—Of a total of 265 patients with neurosyphilis treated with therapeutic vivax malaria during the past year, thirty-five white patients were included in this study. Of these thirty-five patients, only twenty-seven were included in the final analysis of results. Of the eight patients not completely analyzed, three were originally inoculated intradermally and demonstrated negative parasitemias for four weeks following inoculation. On subsequent intravenous inoculation with the same strain of vivax malaria, these patients ran courses typical of susceptible individuals. Two other patients were inoculated with infected mosquitoes (a minimum of two infected mosquitoes positive on dissection), but they also experienced negative parasitemias for four weeks postinoculation. The confusing results in these five patients receiving intradermal and mosquito inoculations were attributed to technical difficulties. The remaining three patients excluded from the study were strongly suspected of illegally consuming atabrine during their hospitalization.

The original vivax inoculation in most instances was performed intravenously, but a few successful mosquito and intradermal inoculations were also employed. Clinical activity following the original inoculation persisted until the occurrence of a spontaneous remission. No therapeutically interrupted original infections were included in this series because of the greater possibility of active recurrence of the original strain later in the patient's hospitalization. In spite of the negative histories of previous malaria attacks in twelve patients,

most of them had some degree of malaria immunity at the onset, with spontaneous remissions occurring prior to the exhaustion of the patient by a prolonged number of paroxysms. Reinoculation and further clinical activity was thus feasible within the therapeutic realm.

Following each spontaneous remission after the original inoculation, interval quinine therapy (10 gr. per 50 pounds of body weight daily for five days) was withheld until the parasitemia decreased to submicroscopic densities. This was merely an added precaution in sterilizing the blood stream prior to reinoculation. From three to five days following the completion of quinine therapy, an adequate interval for the excretion of the drug, reinoculations with either homologous or heterologous vivax strains were accomplished. All reinoculations were performed intravenously, but the total quantitative parasite dosage was varied in order to estimate the effect of the parasite dosage on the prepatent and incubation periods in patients whose immunity was heightened by recent malaria attacks. Following this reinoculation, clinical activity ensued until the development of a second spontaneous remission. Routine atabrine therapy to terminate completely the infection was withheld until the second parasitemia was either negative or at insignificant levels at the end of two weeks following the last bout of clinical activity.

An example of the cross-inoculation technique in an individual case can be summarized from Table I. Patient 153 was originally infected with strain M (*P. vivax* McCoy) and experienced fourteen paroxysms reaching 103° F. or more and twenty-seven days of clinical activity. Following a spontaneous remission, this patient received five days of interval quinine therapy. Three days later, he was reinoculated with strain A (*P. vivax* DuBose). He then experienced five additional paroxysms reaching 103° F. or more and sixteen days of clinical activity. After a second spontaneous remission, routine atabrine therapy was finally instituted.

The Reinfection Index.—Since this study was primarily concerned with the relationship of different vivax strains to each other, it was necessary to fix some factor which would largely cancel the effect of the varied individual malaria immunity prior to original inoculations. For example, if on original inoculation a patient experiences eighteen paroxysms, he is much more susceptible to the disease than the patient who experiences only four paroxysms initially. In the event, however, that each of these patients, on subsequent reinoculation, experiences two paroxysms, it cannot be assumed that the relationship of the reinfection strain to the original strain was the same in both cases, since one's immunity to malaria was much greater than the other's prior to the experiment. A simple method of cancelling this pre-existing individual immunity as a factor in determining strain difference was devised. The number of paroxysms experienced after reinfection was calculated as a percentage of the number of paroxysms experienced after original infection. This percentage figure was designated the *reinfection index*. To follow the previous example, the reinfection index, in the case of the patient experiencing eighteen fevers on original infection

TABLE I.—CROSS-INOCULATION DATA

PATIENT	HOME STATE	STRAIN		HISTORY OF MALARIA	PAROXYSMS			DAYS OF CLINICAL ACTIVITY		
		ORIG.	REIN.		ORIG.	REIN.	R.I.	ORIG.	REIN.	R.I.
347	Ga.	M	M	Malaria, 1935	18	2	11.1	21	4	19.0
153	Ind.	M	A	Negative	14	5	35.7	27	16	59.3
157	Ala.	M	B	Malaria, 1938 (Ala.)	6	4	66.7	8	7	87.5
455	Ga.	M	C	Malaria, 1930 (Fla.)	7	2	28.6	9	3	33.3
349	N. Y.	M	D	Native-born Greek	4	2	50.0	9	3	33.3
442	Ga.	A	M	? Malaria (Panama)	4	10	250.0	9	22	244.0
514	Ky.	A	A	"Chills," 1930 (Ky.)	4	0	0.0	5	0	0.0
398	Vn.	A	B	Negative	15	4	26.7	17	7	41.2
501	S. C.	A	C	? Malaria as child	3	1	33.3	4	3	75.0
418	Ala.	A	D	Negative	8	3	37.5	12	6	50.0
345	S. C.	B	M	Negative	12	4	33.3	22	7	31.8
239	Fla.	B	A	Malaria, 1937 (Fla.)	9	5	55.5	11	5	45.5
384	S. C.	B	B	Negative (but in Southwest Pacific)	12	1	8.3	23	3	13.0
239	Fla.	B	C	Malaria, 1937 (Fla.)	9	8	89.0	11	9	81.8
326	Va.	B	D	Negative	10	8	80.0	16	13	81.3
400	Ohio	C	M	Malaria, ? 1941	13	7	53.8	26	10	38.4
239	Fla.	C	A	Malaria, 1937 (Fla.)	8	5	62.5	9	5	55.5
514	Ky.	C	A	"Chills," 1930 (Ky.)	4	3	75.0	8	5	62.5
261	Texas	C	B	Negative	6	3	50.0	14	7	50.0
424	Ga.	C	C	Malaria, 1935 (Ga.)	11	0	0.0	13	2	15.4
218	Okla.	C	D	Negative	12	3	25.0	24	7	29.2
539	Ala.	C	D	Malaria, 1939 (Ky.)	7	10	143.0	10	20	200.0
397	La.	D	M	? (Paretic)	6	4	66.7	8	7	87.5
294	Texas	D	A	Mexican	3	4	133.3	3	7	233.3
263	Texas	D	B	Negative	1	1	100.0	1	1	100.0
296	N. Y.	D	C	Negative	5	7	140.0	8	9	112.2
397	La.	D	D	? (Paretic)	6	2	33.3	8	3	37.5

Strains of *P. vivax*: M, McCoy; A, DuBose; B, Jenkins; C, Fort; D, Slimes.

Orig., Original infection; Rein., reinfection; R.I., reinfection index (per cent). The figures for paroxysms following inoculation apply to those reaching 103° or more; the days of clinical activity include the time interval from the onset of the first febrile elevation to the cessation of clinical activity for that infection; the number of the patient represents the center number of each patient.

and two on reinfection, would be 11.1. On the other hand, the index would be 50.0 in the case of the patient developing four fevers on original infection and two on reinfection. The higher the reinfection index, therefore, the greater is the difference between the strains cross-inoculated.

Although other studies of malaria immunity base the duration of an individual infection on the days of clinical activity from the onset of elevation in temperature until its permanent cessation, this estimation cannot readily be evaluated in terms of therapeutic adequacy in the treatment of neurosyphilis. The usual criterion of adequate malaria therapy is the actual number of paroxysms reaching a certain febrile height (103° F. or more) rather than the duration of total clinical activity which includes fever-free days. Since this study was undertaken for the purpose of applying the results to therapeutic malaria, the reinfection indices, based on number of paroxysms only, were analyzed. A comparison of the indices by both methods, however, reveals little significant difference (see Table I).

RESULTS

The clinical implications of this cross-inoculation study can be evaluated only after a determination of the immunity characteristics of the series, with a reduction of the patients' varied previous experience with malaria or residence

in endemic areas to a common level. It is our belief that the reinfection index is the single implement most effectively used in judging the results on a common basis. It may be of interest, however, to analyze the series even more closely. Eighteen of the twenty-seven patients (66.6 per cent) experienced less than ten paroxysms on original infection, indicating partial immunity of some type. Only fourteen of the twenty-seven patients had definite previous histories of attacks of natural malaria, but three of those had more than ten paroxysms following original inoculation and apparently had never gained any great degree of immunity from their natural infection. Since Negro patients are highly immune to vivax malaria, only white patients were considered in this study; one, however, was Mexican. Of the thirteen patients with no histories of previous infection, one, whose story was unreliable, had had early paresis.

An analysis of the home states of patients listed in Table I reveals that of the four patients with negative histories who developed partially immune clinical courses, two were from Texas, one was from New York, and one was from Alabama. Three, therefore, may have resided for long intervals in endemic areas. One other consideration was important in evaluating the results. If a patient had experienced a previous attack of natural malaria in one of the Southwest Pacific islands from which our strains were recovered, he might have an adequate infection when inoculated originally with Strain M (Southeast United States), but he might then show practically no clinical activity when reinfected with one of the other strains. In such a case, it would be impossible to distinguish between homologous immunity to the original induced infection or homologous immunity to the original natural attack in the endemic region from which our reinfection strain originated. Confusing as this appeared to make matters, it did not enter extensively into the interpretations, for only one patient had experienced his natural attack outside continental United States, and that was in Panama, not the Southwest Pacific islands.

HOMOLOGOUS IMMUNITY

The mean value (Table II) for the duration of paroxysms following original infection in the five patients reinfected with homologous strains was 10.2. The duration of paroxysms in this group on reinfection averaged 1.0. These figures alone denote the characteristics of homologous immunity, with reinfection with

TABLE II. SUMMARY OF MEAN VALUES

Patients reinfected with homologous strains (five)	
Duration of paroxysms following original infection	10.2
Duration of paroxysms following reinfection	1.0
Reinfection index	10.5
Patients reinfected with heterologous strains (twenty-two)	
Duration of paroxysms following original infection	7.5
Duration of paroxysms following reinfection	4.5
Reinfection index	74.3
Individual heterologous reinfection indices of each strain	
M vs. A, B, C, D	73.1
A vs. M, B, C, D	78.8
B vs. M, A, C, D	62.6
C vs. M, A, B, D	70.0
D vs. M, A, B, C	86.1

similar strains producing practically no further clinical activity. The homologous reinfection index, mean value, was 10.5. Practically applied, this would mean that rarely would one expect to induce a total reinfection with homologous strains which would last for more than one-tenth of the duration of the original infection. The use of homologous strains in completing therapeutic vivax malaria in patients demonstrating partial immunity is, therefore, not practicable.

HETEROLOGOUS IMMUNITY

The mean value (Table II) for the duration of paroxysms following original infection in the group of twenty-two patients reinfected with heterologous strains was 7.5. However, the duration of paroxysms following reinfection with these heterologous strains was 4.5. Thus, in spite of the fact that the group as a whole displayed a greater degree of pre-existing malaria immunity, they developed more prolonged reinfection clinical activity than did the group reinfected with homologous strains. The heterologous reinfection index (mean value, 74.3) clarifies this differentiation. The reinfection of a previously vivax-infected patient with a heterologous strain would, on the average, produce clinical paroxysms totaling approximately 75 per cent of the clinical paroxysms experienced on original infection. Further analysis reveals that fifteen of the twenty-two (68.2 per cent) reinfected with heterologous strains showed secondary clinical activity greater than 50 per cent of the primary course. Four of the twenty-two (18.2 per cent) showed from 31 to 40 per cent reactivity and three of the twenty-two (13.6 per cent) between 25 and 30 per cent. If heterologous strains of vivax malaria were, therefore, used to reinfest patients experiencing partial immunity (Group B, from 5 to 8 paroxysms) following original infection, 68.2 per cent would experience therapeutic courses totaling more than 50 per cent of their original bouts and 86.4 per cent more than 31 per cent of their original courses.

Table II lists the mean values of the reinfection indices when heterologous reinfection was employed against each single strain in turn. The figures varied from 62.6 to 86.1. It cannot be said with certainty, therefore, that any one of the strains was very much more active than any other in heterologous reinfection. Clinical observations, however, indicated that strain A (DuBose) and strain D (Siimes) were the most potent in producing severe infections with longer, more intense paroxysms. Extensive experience with Southwest Pacific strains during the past year has disputed the common impression that they are more virulent than the United States strains utilized as therapeutic agents. Complications encountered during therapeutic malaria with these two types of *P. vivax* have been strictly comparable.

The relationship of the reinoculation intravenous parasite dosage to the duration of the prepatent and incubation periods of the reinfection can be studied from the tabulation of mean and median values listed in Table III. Included as a comparison against the twenty-one heterologous cross-inoculation cases is a control series of seventy-eight patients, all of whom experienced completely susceptible courses of vivax malaria. It is first noted that, in partially

TABLE III. RELATION OF INOCULATION PARASITE DOSAGE TO PREPATENT AND INCUBATION PERIODS

PARASITE DOSAGE	CONTROL GROUP						CROSS-INOCULATION GROUP					
	NUMBER OF PATIENTS	P.P. PER		INC. PER			NUMBER OF PATIENTS	P.P. PER		INC. PER		
		ME.	MD.	ME.	MD.			ME.	MD.	ME.	MD.	
0 to 10 mil.	35	6.8	6.0	7.6	7.0		7	3.7	3.0	6.6	6.0	
11 to 30 mil.	20	3.3	3.0	3.8	3.0		7	6.4	7.0	11.9	12.0	
31 to 150 mil.	23	2.9	3.0	2.5	2.0		7	3.1	3.0	8.0	9.0	

P.P. Per., Prepatent period; Inc. Per., Incubation period; Me., mean (average); Md., median; mil., millions.

immune individuals, the incubation period is invariably longer than the prepatent period, the difference varying from three to six days in this series. This confirms the fact that clinical activity in a partially immune patient begins only after the parasite density attains high levels and rarely when the density is submicroscopic. This is in contrast to the control series of susceptible patients, where the prepatent and incubation periods are of almost equal duration. Further examination reveals that in susceptible patients, the prepatent and incubation periods vary inversely with the inoculation parasite dosage, with the longest periods following the smallest inocula and the shortest periods the largest inocula. This relationship, however, is not maintained in any group of patients with varying degrees of immunity, since such immunity predetermines to some extent the duration of these periods. This is indicated statistically in Table III (cross-inoculation group), in which it is noted that longer prepatent and incubation periods occur with higher parasite dosages (from 11 to 30 millions) than with lower dosages (from 0 to 10 millions).

SUMMARY

The problem of partially immune responses to vivax inoculation in white patients with neurosyphilis who have no previous history of attacks of natural malaria has been a provoking one. These patients, part of an ever-increasing group occasioned by the prolonged residence of soldiers in hyperendemic overseas areas, cannot readily be screened out for inoculation with other species of malaria because of their negative histories. They may, therefore, require reinoculation for the completion of therapeutic courses.

The present custom of reinoculating these patients with quartan malaria raises two objections: (1) Quartan reinoculation materially prolongs hospitalization and morbidity because of the *prolonged incubation period and the longer duration and cycle of paroxysms* and (2) quartan infections, differing from the other species of malaria, often become latent for many years and may recur or act as the source of accidental transfusion malaria.

Homologous immunity is characterized by the production of minimal clinical activity in patients reinfected with the same or similar substrain of vivax malaria. The duration of the homologous reinfection in this series of patients averaged 10.5 per cent (reinfection index) of the duration of the original infection with that same strain.

Heterologous immunity exists when reinfection of a patient with a strain of *P. vivax* differing in geographic origin from the original strain results in definite clinical activity sufficient for the completion of a course of malaria therapy. In this cross-inoculation study, the duration of the heterologous reinfection averaged 74.3 per cent (reinfection index) of the duration of the original infection.

In a control series of seventy-eight susceptible patients, intravenous inoculation with various parasite dosages of *P. vivax* revealed that the subsequent prepatent and incubation periods varied inversely with the total quantitative dosage. This correlation, however, does not apply to any series of patients with immunity to malaria.

It seems desirable, as a result of malaria immunity studies, to reinoculate with heterologous strains* of *P. vivax* those white patients with neurosyphilis experiencing partially immune types of original infection (from five to eight paroxysms). Adequate completion of therapeutic courses and avoidance of unnecessary quartan infections thus can be accomplished.

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*Heterologous Southwest Pacific strains of *P. vivax* are currently available at the Florida State Hospital, Chattahoochee, Fla.

BACTERIOLOGIC STUDIES OF THE SPUTUM IN PATIENTS WITH PNEUMOCOCCAL PNEUMONIA TREATED WITH PENICILLIN

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A STUDY of the sputum of patients with pneumonia often provides a means of following the bacteriologic response to various forms of therapy and gives some insight into the mechanism of action of the therapeutic agents. Changes in the sputum may reflect to a considerable degree those which occur in the lung. Such studies have previously been made, chiefly by Frisch,¹⁻¹² in some patients receiving only supportive therapy and in others who were treated with specific antisera or sulfonamide drugs.

The result of various forms of effective therapy is a reduction in the number of organisms in the sputum and in the lungs. In untreated patients, agglutination of pneumococci in the sputum is sometimes, though not always, observed between the sixth and tenth day of the disease. Adequate therapy with type-specific antipneumococcus serum brings about the agglutination of pneumococci in the sputum within from six to twelve hours. This is usually followed by phagocytosis and by a gradual reduction in the number of organisms over a period of several days. The sulfonamides exert a direct bacteriostatic effect which usually results in a prompt reduction in the number of pneumococci within from twelve to thirty-six hours. There is, however, considerable variation in the persistence of pneumococci in sulfonamide-treated patients, and they persist for long periods in about 30 per cent or more of patients so treated.^{13, 14} The persistence of pneumococci during the sulfonamide therapy is not related to the failure of the patient to develop homologous antibodies.¹⁴ It may be associated with the development of resistance of the pneumococci to sulfonamides as measured by *in vitro* tests,⁶ although this, too, is only rarely the case.¹⁴

Frisch and his associates also correlated the numbers of pneumococci found in Wright-stained smears of sputum with the prognosis and with other significant clinical and laboratory findings.^{9, 10} He found that the numbers of pneumococci are directly proportional to the case mortality and to the incidence of bacteremia and leucopenia.

Penicillin is much more effective than sulfonamides against pneumococci *in vitro* and probably has a different mechanism of action. It was of interest, therefore, to study the changes in the pneumococci and other organisms in the sputum of patients under treatment with this antibiotic. The present paper deals with observations on the sputum of twenty-six patients with pneumococcal pneumonia treated with penicillin.

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MATERIALS AND METHODS

Selection of Patients.—The twenty-six patients studied were admitted to the medical wards of the Boston City Hospital between December, 1944, and April, 1945. They ranged in age between 16 and 88 years and twelve of them were over 50 years old. All were acutely ill with lobar pneumonia and produced sputum in which pneumococci were identified by Neufeld typing at the first examination. All of these patients denied previously receiving sulfonamide drugs. Blood taken on admission from twenty-two of these patients was tested for the presence of sulfonamide, and no measurable concentration was found.

Penicillin Therapy.—Penicillin therapy was begun on the third day or earlier in seventeen patients, between the fourth and sixth day in six, and on the tenth day of illness in the remaining three. The average duration of the disease at the onset of therapy was 3.5 days. Commercial penicillin (G) was given intramuscularly in most instances, but in two patients penicillin X was used. In two others oral penicillin was given, and one patient received penicillin G by inhalation. The intramuscular injection consisted of 15,000 or 20,000 units given every two hours for the first day followed by these amounts every three hours for another two or three days, after which the dose was often reduced to 10,000 units every three hours. Treatment was discontinued after three days or less in three patients, after from four to six days in seventeen, and after seven days or longer in the remaining six. The average duration of treatment was 5.7 days. The oral penicillin was given in doses of 100,000 units every two hours at first and later every three hours. The nebulized penicillin was given in doses of 20,000 units every two hours. Sulfonamides were used in only two patients for complicating infections some time after the penicillin was stopped and the sputum studies were discontinued.

Bacteriologic Studies.—Blood cultures were made on every patient before treatment was started and were repeated at irregular intervals when indicated. Sputa were obtained before the first dose of penicillin and at suitable intervals during and after treatment. These sputa were subjected to the following studies: (1) Direct examination of the sputum was made by the Neufeld method both for the different types of pneumococci and the relative numbers of each type. (2) Gram- and Wright-stained preparations were examined by the method of Frisch for the number of characteristic lancet-shaped diplococci, the presence of chaining, phagocytosis, and reticulation. (3) A characteristic portion of the sputum was homogenized, and from it streaks and substreaks were made in progression on the quadrants of the surface of horse blood agar plates in order to observe roughly the relative numbers of different bacteria, including pneumococci, beta and alpha hemolytic streptococci, staphylococci, *Hemophilus influenzae*, and *Hemophilus hemolyticus*. Identification was mostly by morphology, but all pneumococci were typed, staphylococci were tested for coagulase activity, and green streptococci were tested for bile solubility. (4) Serial tenfold dilutions in plain broth were also made from the homogenized sputum, and blood agar plates were poured with from 10^{-4} to 10^{-9} c.c. Estimations of the numbers of typical pneumococcus colonies were made,

and doubtful colonies were subcultured and identified by bile solubility and Neufeld typing. (5) Whenever pneumococci could not be identified directly in the sputum by the Neufeld method, some of the sputum was inoculated into a mouse, and the peritoneal exudate and heart blood were later examined for pneumococci.

Throat cultures were made in many instances at the time of the initial sputum and later whenever sputum was not obtainable. Streaks were made on the surface of blood agar plates from the swab or from broth into which the swab was immersed and treated like the sputum. Swabs were then used only for the study of the presence and identity of pneumococcus types.¹⁵ Blood broth containing the swab was incubated from four to eight hours until a uniform growth occurred, after which it was examined by the Neufeld method, and a small amount was injected into a mouse. The peritoneal exudate and cultures of the heart blood were subsequently examined for pneumococci.

The sensitivity to penicillin of most of the strains of pneumococci that were isolated, and of many of the other organisms, was tested by the method of Rammelkamp and Maxon.¹⁶

RESULTS

A summary of certain relevant features of the cases studied in relation to the persistence of the pneumococci is given in Table I. The cases were of moderate severity; multiple lobes were involved in nine of the patients and blood cultures were positive in ten (38 per cent) at entry.

A total of 154 sputa were studied in the twenty-six patients and, in addition, forty-five throat cultures were made in twenty patients, making an average of almost eight specimens from each patient. Specimens were obtained throughout the hospital stay of the patients and, in a few instances, during follow-up visits. The last culture was made on or before the tenth day in fourteen patients, between the eleventh and twenty-first day in nine, and between the twenty-third and thirtieth day in the remaining three. The average period of observation in the twenty-four patients who recovered was thirteen days.

A total of forty-two strains of pneumococci were isolated and identified in the twenty-six cases. A single type was obtained in fourteen of the cases, two types were obtained from each of ten cases, and three and five types, respectively, were identified in the two remaining cases. Nineteen of the strains were of the types commonly associated with primary lobar pneumonia, namely types 1, 2, 4, 5, 7, and 8. Of the remaining strains, five were type 3, one was type 6, and the others were of the so-called "higher" types. In each of the twelve cases with multiple types, one of the strains of pneumococci was of the common types.

Persistence of the Pneumococci Present Before Penicillin Treatment Was Started.—The number of days during which the pneumococci present in the original sputum persisted after treatment with penicillin was started are shown in Table II. Only one of every six of these strains persisted throughout the period of observation, and twenty-one of the thirty-five strains (60 per cent)

TABLE I. OCCURRENCE AND PERSISTENCE OF PNEUMOCOCCI IN TWENTY-SIX PENICILLIN-TREATED PATIENTS WITH PNEUMONIA

CASE	BLOOD CULTURE*	LOBES INVOLVED†	PENICILLIN THERAPY			PNEUMOCOCCUS TYPE	PNEUMOCOCCI IN SPUTUM				CLINICAL RESPONSE, COMPLICATIONS, AND REMARKS
			DAY BE-GUN‡	TOTAL DOSE			SENSITIVITY	DAYS FOUND¶		LAST TEST	
				UNITS X 1,000§	NUMBER OF DAYS			FIRST	LAST		
1	7	Rl, Ll	2	515	5	7	0.016**	0	23	23	Crisis 24 hours; chronic cough
2	0	Ll	1	570	5	1	0.016	0	3	8	Lysis 3 days
3	0	Ll, Rl	10	265	1½	3	0.016	0	½	1	Died 1½ days; moribund on entry; 88 years old
4	0	Ruml, Ll	3	785	8	12	0.016	0	3	11	Lysis from 2 to 5 days; frequent colds
5	1	Ll	4	520	5	1	0.031	0	1	10	Crisis 36 hours; alcoholism
6	0	Rm	1	385	4	2	0.016	0	2	4	Crisis 48 hours
7	1	Rum	3	1,530	10	1	0.008	0	7	15	Lysis 3 days; moribund on entry; 500 colonies per cubic centimeter of blood; W.B.C. 400; polymorphonuclears, 1%; alcoholism
8	4	Rl	4	740	6	4	--	0	12	17	Lysis 4 days; chronic pyelonephritis
9	1	Ll	3	660	6	1	--	0	6	8	Lysis from 2 to 7 days; low-grade fever and pleuritic pain 12 days
10	0	Ll	3	415	5	7	0.016	0	8	8	Crisis 24 hours
11	0	Rl	2	965 X	8	28	--	0	7	7	Lysis 9 2 days; fever from active tuberculosis (positive sputum)
12	0	Ll	1	455 X	5	8	0.031	0	7	7	Crisis 12 hours
13	7	Ll	2	6,390 p.o.	6	7	0.031	0	3	12	Lysis 5 days
14	0	Ll	2	880 inh.	6	12	0.016	0	7	14	Crisis 24 hours
15	0	Rum	10	670	6	1	0.031**	0	14	14	Crisis 24 hours; temperature of 100° F. for 8 days
						43	0.016	12	12		Crisis 24 hours
16	0	Ll	2	525	4	3	0.031	0	2	5	Crisis 12 hours; pneumonia (? etiology) each of last 3 winters
17	0	Ruml	3	525	5	7	0.016	0	2	8	Crisis 24 hours
						6	--	2	2		
18	0	Ll	1	320	3	5	0.031	0	2	11	Crisis 24 hours
19	1	Ruml	6	1,950	10	13	--	1	1		
						1	0.031**	0	9	30	Persistent fever; 1 interlobar effusion; staphylococcus (sensitivity, 8 units) persisted
						19	--	0	9		
20	0	Rl	2	500	6	3	0.008	0	4	21	Crisis 12 hours; delirium tremens
21	1	Ll	10†	450	3	11	0.031	4	12	2	Died third day; hemiplegia and coma
						9	--	0	½		
22	0	Ll, Rl	1	595	5	3	0.016	0	½	9	Lysis 3 days; "shock" on entry; asthma; bronchiectasis; congestive heart failure
						14	--	0	0		
						44	0.016	0	2		
23	1	Ll	4	425	4	1	0.016	0	4	6	Crisis 24 hours; alcoholism
						8	--	1	1		
						18	0.016	1	1		
						19	0.031	0	1		
						44	0.016	0	½		
24	0	Rl	6	940	9	3	0.008	0	½	25	Lysis 3 days; chronic cough
						17	0.016	0	2		
25	0	Ll	1	555	4	8	0.016	0	1	9	Crisis 24 hours; "pleurisy" 2 years before
						20	0.016	2	2		
26	7	Ruml	5	8,505 p.o.	9	7	0.008	0	2	19	Lysis from 2 to 5 days; 1 interlobar effusion
						19	--	0	1		

*Numbers represent types of pneumococci and 0, no growth.

†R, Right; L, left; u, upper; m, middle; l, lower.

‡Refers to day of disease.

§X, Intramuscular penicillin X; p.o., oral; inh., by inhalation; others received intramuscular commercial penicillin.

¶Minimum concentration of penicillin (units per cubic centimeter) required to sterilize a culture inoculated with from 1,000 to 10,000 pneumococci per cubic centimeter in eighteen hours; --, not done.

||Refers to day after onset of treatment; 0, specimen obtained before treatment was given.

**Same result in last specimen.

TABLE II. PERSISTENCE OF THIRTY-FIVE STRAINS OF PNEUMOCOCCI FOUND BEFORE PENICILLIN TREATMENT WAS STARTED

LAST POSITIVE CULTURE	NUMBER OF STRAINS
First day	11
Second day	7
Third day	3
Fourth day	2
Sixth to twelfth day	6
Total	29 (83%)

Average persistence of all thirty-five strains, 4.3 days.

Average persistence of the twenty-nine strains which disappeared during the period of observation, 3.0 days.

TABLE III. SIX STRAINS OF PNEUMOCOCCI WHICH PERSISTED THROUGHOUT PERIOD OF OBSERVATION

CASE	DAYS OF PENICILLIN THERAPY	PNEUMO-COCCUS TYPE	PENICILLIN SENSITIVITY	DAYS POSITIVE	COMPLICATIONS
1	5	7	0.016*	23	Chronic bronchitis
10	5	7	0.016	8	
11	8	28	----	7	Tuberculosis (sputum positive)
12	5	8	0.031	7	
15	6	1	0.031*	14	
16	4	20	0.031	5	

*Same for original strain obtained before treatment and for the strain obtained twelve days later.

were found for only three days or less. The average time that elapsed between the beginning of penicillin treatment and the last time these pneumococci were found was 3.0 days. Twenty-six of the twenty-nine strains could no longer be identified in sputum or throat cultures before the penicillin therapy was completed.

A summary of the relevant findings concerning the six strains which persisted throughout the period of observation is shown in Table III. Five different types were represented. Treatment with penicillin was given for from four to eight days in these cases and they were studied for from five to twenty-three days (average 10.7 days). The strains were similar in penicillin sensitivity to those which disappeared early. In two instances strains of the same type isolated before treatment and again twelve days later were compared, and no change in sensitivity to penicillin could be demonstrated. Two of the patients from whom these strains were obtained had chronic respiratory infections. In three of the patients the strains of pneumococci had disappeared temporarily only to reappear in later cultures.

Appearance of Additional Strains of Pneumococci During or After Penicillin Therapy.—Seven strains of pneumococci appeared in six patients after penicillin therapy was started, five during the treatment and two after it was stopped. Six of these strains were identified in only one specimen and the remaining one was first noted on the fourth day of treatment and persisted over an eight-day period. None of them was present in the last culture. One of the strains was type 8, and the others were of higher types. The penicillin sensitivity of the new strains was compared with that of the original ones in four

TABLE IV. PENICILLIN SENSITIVITY OF STRAINS OF PNEUMOCOCCI AT TIME OF THEIR ORIGINAL ISOLATION

MINIMUM INHIBITING CONCENTRATION OF PENICILLIN	NUMBER OF STRAINS	NUMBER OF STRAINS WHICH PERSISTED THROUGHOUT THE PERIOD OF OBSERVATION
0.008 units per cubic centimeter	4	0
0.016 units per cubic centimeter	17	2
0.031 units per cubic centimeter	11	3
Total	32	5

eases. These were found to be the same in two instances (Cases 23 and 25); the new strain was twice as sensitive as the original one in Case 15, and the original strain was four times as sensitive as the new one in Case 20.

Sensitivity of the Pneumococci to Penicillin.—Thirty-two strains of pneumococci were isolated when first encountered and were tested for sensitivity to penicillin. Twenty-eight of these strains were obtained before treatment was started, three were isolated during penicillin therapy, and one was isolated after it was discontinued. The results are shown in Table IV. All were quite susceptible, most of them requiring two or four times as much penicillin as a control strain of hemolytic streptococcus, No. 98, which was sensitive to 0.008 units per cubic centimeter of culture. Three additional strains of the same type were isolated again, one after four days of treatment and the other two after eight days, and all were just as sensitive as the original strains. The strains isolated during or after therapy were within the same range of sensitivity as those isolated before treatment was started. There is a slight suggestion that the less sensitive strains tended to persist longer, but the number of strains and the range of their sensitivity were too small for any definite conclusion.

Clinical Response to Treatment.—Among the patients who recovered, twelve were essentially afebrile and free of acute symptoms within forty-eight hours after the first dose of penicillin, while in nine others some fever persisted for from three to five days, although the patients had shown symptomatic improvement earlier. In three patients low-grade fever persisted for eight, twelve, and twenty-eight days, respectively; the first two of these patients had no complications, but the third developed a staphylococcal pneumonia and probably some interlobar effusion.

There were two deaths. One occurred (Case 3) in a man 88 years old with type 3 pneumococcal pneumonia involving both lower lobes. The blood culture taken on the tenth day of the disease and before treatment was started was negative. Pneumococci could not be found in the last sputum which was taken twenty-four hours after penicillin was started, and the patient died twelve hours later. The second patient (Case 21) was 65 years old and had a type 1 pneumococcal pneumonia and bacteremia. He became comatose, developed a hemiplegia, and died on the third day. Pneumococci could not be found in the throat culture taken shortly before death.

A summary of the persistence of pneumococci in relation to the duration of fever after treatment was started is shown in Table V. There was no defi-

TABLE V. RELATION OF RAPIDITY OF CLINICAL RESPONSE AND PERSISTENCE OF PNEUMOCOCCI FOUND BEFORE TREATMENT WAS STARTED

DAYS SPUTUM POSITIVE	RECOVERED WITHIN 48 HOURS	FEVER FROM 3 TO 5 DAYS	FEVER 8 DAYS OR MORE	DIED
1 to 3	7	11	--	3
4 to 10	7*	1	3	-
More than 10	1†	1	1‡	-
Strains	15	13	4	3
Cases	12	9	3	2

*Last culture positive on fifth day.

†Last culture positive on twenty-third day.

‡Last culture positive on fourteenth day.

TABLE VI. RELATION OF BACTEREMIA TO PERSISTENCE OF PNEUMOCOCCI UNDER PENICILLIN TREATMENT

DAYS SPUTUM POSITIVE	NUMBER OF CASES WITH BLOOD CULTURE	
	POSITIVE	NEGATIVE
3 or less	4	9
4 to 10	4	6†
More than 10	2*	1‡
Total cases	10	16

*Last culture positive on twenty-third day in one case.

†Last culture was positive in four cases.

‡Last culture positive on fourteenth day.

nite correlation except among the three cases in which the fever continued the longest. Among these the pneumococci persisted more than three days in each instance.

Bacteremia.—There were ten patients in whom positive blood cultures were obtained before penicillin therapy. Type 1 pneumococci were obtained in six patients, type 7 in three, and type 4 in one. The persistence of pneumococci in these patients compared with that in the patients with negative cultures is shown in Table VI. There is a suggestion of a longer persistence of pneumococci in the sputa of the patients with bacteremia, but the numbers of these patients are too small and the correlation is not striking. Case 7 is of interest in this respect. The patient had an overwhelming bacteremia with about 500 colonies of type 1 pneumococci grown per cubic centimeter of blood before treatment, and the leucocyte count at the time was 400 per cubic millimeter of blood, of which only 1 per cent were polynuclear cells. This patient showed steady and dramatic improvement under penicillin therapy. The second blood culture taken after ten hours was sterile, but pneumococci persisted in the sputum for seven days and could not be found thereafter.

Effect of Chronic Respiratory Infections and Previous History of Pneumonia.—There were five patients who gave a history of chronic cough of more than a few months' duration. In only one of them (Case 1) did pneumococci persist after treatment. Five additional patients had other chronic respiratory complaints: one had frequent upper respiratory tract infections, one had asthma, one had recurrent pleurisy, one had active pulmonary tuberculosis, and one had an attack of pneumonia each of the three preceding winters. The only one of these five patients in whom pneumococci persisted during seven days of

TABLE VII. RELATION OF CHRONIC RESPIRATORY SYMPTOMS TO PERSISTENCE OF PNEUMOCOCCI IN SPUTUM AFTER PENICILLIN THERAPY

DAYS SPUTUM POSITIVE	PATIENTS WITH CHRONIC RESPIRATORY SYMPTOMS	PATIENTS WITHOUT CHRONIC RESPIRATORY SYMPTOMS
3 or less	7	6
4 to 10	2*	8‡
More than 10	1†	2§
Total cases	10	16

*Last culture positive seventh day in one patient.

†Last culture positive twenty-third day.

‡Last culture positive in three patients.

§Last culture positive in one patient.

observation was the one with active tuberculosis (Case 11). Pneumococci were found after the third day in only one additional patient. A comparison of the persistence of pneumococci in the patients with and without chronic respiratory symptoms is given in Table VII. There was no correlation in this small group of patients.

Effect of Complications.—As might be expected in penicillin-treated cases, purulent pneumococcal complications were not recognized in the present cases. One patient (Case 19) developed a complicating staphylococcal pneumonia and signs of an effusion. Signs of a small interlobar effusion were also made out in another patient (Case 26), but the presence of fluid was not proved by thoracentesis in either of these two patients. The occurrence of agranulocytosis before treatment was started in Case 7 has already been mentioned, and this patient also suffered from alcoholism and a toxic psychosis. Pneumococci persisted for nine, two, and seven days, respectively, in these three patients.

Significance of the Numbers of Pneumococci in the Initial Sputum.—Quantitative bacteriologic studies of sputum and throat cultures are notoriously unsatisfactory and involve tremendous errors. When a single organism is greatly predominant or is present in almost pure culture, as is sometimes the case in severe pneumonia, such studies may be carried out successfully. When a variety of organisms are found in relatively large numbers, as they usually are after therapy with active chemotherapeutic or antibiotic agents, the quantitative aspects are not very reliable. Only a rough estimation then can be made of the relative numbers of the different organisms present and even this involves considerable errors. In general, it has not been possible to differentiate the individual colonies of organisms in poured blood agar plates of sputum containing more than 10^{-5} c.c., and more often 10^{-6} or 10^{-7} c.c. was the largest amount that proved workable. Even smaller amounts must sometimes be used if individual colonies are to be picked and identified.

In Table VIII is listed the number of patients in whom from two to ten pneumococcus colonies were recognized in pour plates of various dilutions of the original sputum obtained before penicillin was started. This is compared with the numbers of pneumococci seen in Wright- and Gram-stained smears of the same sputum and is also correlated with some of the more important features of the disease. The number of cases is too few for any definite deductions. It does seem, however, that there was a fairly good correlation between

TABLE VIII. ROUGH QUANTITATION OF NUMBERS OF PNEUMOCOCCI IN SPUTUM OBTAINED BEFORE PENICILLIN THERAPY CORRELATED WITH CERTAIN IMPORTANT FEATURES OF PNEUMONIA

GREATEST DILUTION SHOWING PNEUMOCOCCI	NUMBER OF CASES	PNEUMOCOCCI PER HIGH-POWER FIELD IN WRIGHT-STAINED SMEARS			CASES WITH POSITIVE BLOOD CULTURES	MULTIPLE- LOBE INVOLVEMENT	LEUCOCYTES PER CUBIC MILLIMETER		CLINICAL RESULT		
		MORE THAN 100	50 TO 100	LESS THAN 50			5,000 TO 10,000	LESS THAN 5,000	CRISIS LESS THAN 48 HOURS	LYSIS 3 DAYS OR MORE	DIED
10 ⁻⁹	3	3	0	0	2	2	2	1	0	2	1
10 ⁻⁸	11	3	1	7	4	4	2	2	6	4	1
10 ⁻⁷	10	1	1	8	4	3	2	0	3	5	0
10 ⁻⁶ or 10 ⁻⁵	2	0	0	2	0	0	1	1	1	1	0
Total	26	7	2	17	10	9	7	4	12	12	2

the number of pneumococci per cubic centimeter of sputum as determined in the pour plates and the number of characteristic diplococci seen per high-power field in the Wright-stained preparations. Little can be said of the other features. Bacteremia, multiple lobe involvement, and leucopenia tended to occur in patients with large numbers of pneumococci, but this does not show very clearly from the present data. The two patients who died had large numbers of pneumococci, although the deaths were associated with other factors. Following treatment, the pneumococci which persisted were found only in the pour plates of smaller dilutions, and very few were seen in the smears.

The relation of the number of pneumococci observed in the original sputum and their persistence after treatment is roughly shown in Table IX. Within the limits of the method, there seemed to be little correlation between the numbers of pneumococci counted in the pour plates and their persistence except that the patients in whom the organisms persisted for more than ten days all originally had large numbers. The correlation between the numbers of pneumococci seen in the original smears and the persistence of pneumococci was somewhat better but still not very striking.

TABLE IX. PERSISTENCE OF PNEUMOCOCCI IN RELATION TO NUMBERS FOUND BEFORE THERAPY

APPROXIMATE NUMBER OF PNEUMOCOCCI	NUMBER OF DAYS PNEUMOCOCCI PERSISTED IN THE SPUTUM			TOTAL NUMBER OF CASES
	3 OR LESS	FROM 4 TO 10	MORE THAN 10	
Per cubic centimeter of sputum:				
10 ⁹	1	1	1	3
10 ⁸	6	2	3	11
10 ⁷	6	4	0	10
10 ⁶ or 10 ⁵	-	2	-	2
Per high-power field (Smears):				
More than 100	3	2	2	7
From 50 to 100	-	1	1	2
Less than 50	10	6	1	17
Total cases	13	9	4	26

Recognition of Pneumococci.—Throughout this study there was no difficulty in recognizing pneumococci and identifying their type regardless of whether they were found before treatment was begun, during penicillin therapy, or after it was discontinued. When sputum was available and pneumococci were present in the cultures, they were also usually identified directly in the sputum by the Neufeld method, characteristic colonies were observed on the surface of blood agar plate cultures, and the sputum caused the death of mice with multiplication of the organisms in the peritoneal exudate and invasion of the blood stream. Quantitative tests for the virulence of the pneumococci for mice were not done.

Occurrence of Phagocytosis, Chaining, and Morphologic Alterations of the Pneumococci.—In the present studies no definite relationship was noted between the occurrence of chaining and phagocytosis of the pneumococci in the stained smears of the sputum and the duration of the disease or of penicillin treatment or the persistence of pneumococci in the sputum. Chaining was observed in only eight cases, was transient, and was not prominent in any of the specimens.

Phagocytosis was marked in only a few instances. Phagocytosis was first noted between the first and seventeenth day of the disease:—on or before the third day in ten patients, between the fourth and seventh days in eight, on the eighth day or later in five, and not at all in three. In fourteen patients the phagocytosis was first noted before penicillin therapy was started; in six it was seen on the first day of treatment; in two others it was first observed on the third or fourth day of therapy; and in one patient it was noted only eight days after treatment was stopped.

Some morphologic changes in the pneumococci were observed in the smears made after penicillin had been given. In several instances the pneumococci assumed bizarre shapes, took the stain poorly, and appeared to be fragmented in the Wright- or Gram-stained smears of sputum. Similar changes in bacteria subjected to the action of penicillin have been noted by Gardner,¹⁷ and they have also been seen in smears of empyema fluid after local penicillin instillation.¹⁸

Reticulation, as described by Frisch,⁵ was noted in Wright-stained preparations of three of the type 3 cases, in two type 7 cases, and in one type 8 case. It was not marked in any of these cases. The last sputum culture was positive in each of the type 7 and type 8 cases but not in the type 3 cases.

Occurrence of Organisms Other Than Pneumococci.—During penicillin therapy the number of organisms which grew in pour plates of sputum, or on the surface of blood agar plates streaked with sputum, characteristically declined. A few colonies of *Staphylococcus albus* and moderate numbers of alpha hemolytic or nonhemolytic streptococci persisted in almost every case. In twenty-one of the cases small Gram-negative bacilli having the morphology and colony characteristics of *H. influenzae* appeared and usually became numerically the predominating organisms during the course of therapy. *H. hemolyticus* appeared in the sputum of ten patients but did not become predominant in any of them. Beta hemolytic streptococci were found in only five patients, usually in very small numbers and before treatment was started. They disappeared rapidly and completely soon after penicillin was given. Friedländer's bacillus, type A, was present in the sputum of one patient.

Case 19 is an interesting one from the point of view of the occurrence and fate of different types of organisms in the course of therapy. On admission, type 1 pneumococci were grown in the blood culture and types 1 and 19 pneumococci were found in the sputum. The pneumococci disappeared gradually from the sputum after nine days of penicillin therapy. A coagulase-positive and hemolytic *Staphylococcus aureus* first appeared twelve hours after penicillin was started and became the predominant organism. It persisted throughout the hospital course, although there was a definite reduction in the numbers of this organism after sulfadiazine was started. *H. influenzae* also appeared and persisted but in smaller numbers than the staphylococcus. The penicillin sensitivity of the type 1 pneumococcus in this case was 0.031 unit per cubic centimeter, that of the influenza bacillus was 0.5 unit per cubic centimeter, and that of the staphylococcus was from 4 to 10 units per cubic centimeter. The staphylococcus was isolated and tested for sensitivity to penicillin on four

separate occasions with similar results each time. It is of interest, therefore, that in this patient the pneumococcus, which was the most sensitive to penicillin, disappeared first, and the staphylococcus, which was the most resistant of the organisms, persisted the longest.

The penicillin sensitivity of some of the strains isolated in the course of this study have been included in a previous report.¹⁹ Of special interest were seven strains of *H. hemolyticus* which were similar in penicillin sensitivity to most meningococci and staphylococci. The most resistant of these strains required 0.25 unit per cubic centimeter. One strain was sensitive to 0.125 unit, four were sensitive to 0.063 unit, and one was sensitive to 0.031 unit per cubic centimeter.

DISCUSSION

It is appreciated that the number of patients available for the present study was much smaller than was desired. The marked general reduction in the incidence of pneumococcal pneumonia, coupled with the difficulty of finding such patients who arrive in a general hospital before they have been given some antibacterial therapy, is in large part responsible for the paucity of cases. Furthermore, the follow-up in these patients was relatively short due chiefly to the rapid improvement in their clinical condition and consequently their brief period of hospitalization. The cough and sputum in these patients cleared up quite rapidly, although low-grade fever often persisted for several days.

There was little difference in the bacteriologic response of the patients treated with penicillin and those previously studied in this clinic under sulfonamide therapy.¹⁴ Penicillin acts somewhat more rapidly than do the sulfonamides in vitro, but in the patients the two agents seem to cause disappearance of pneumococci from the sputum with equal rapidity. (The pneumococci did, however, clear more rapidly from the sputum after penicillin.²⁰) The differences were not very striking in this small group of patients. Penicillin appears to be more effective than the sulfonamides in preventing purulent pneumococcal complications, and the same mechanism may also have resulted in a smaller percentage of patients in whom pneumococci persisted under penicillin treatment. Only six strains, or 17 per cent of the thirty-five which were originally present, persisted after penicillin therapy, as compared with 30 per cent among the sulfonamide-treated patients previously studied in this clinic.¹⁴ A similar proportion, namely three-fourths of the strains which cleared during therapy, had already disappeared by the third day of treatment with either agent.

The dosage used in the present series of patients was considered to be adequate although others may consider it only moderate. It was sufficient to maintain levels of penicillin in the blood above those required by the sensitivity of the pneumococci, and it seemed adequate clinically. It is possible, however, that larger doses which may permit greater penetration into infected foci might have cleared the pneumococci more rapidly and more completely. The possibility is further suggested by the results obtained in beta hemolytic streptococcal respiratory infections in which the organisms are more sensitive to penicillin and in which similar doses result in more rapid clearing of the

organisms from the pharynx.²¹ The reason for the failure of pneumococci to disappear from the sputum in some patients, in spite of their susceptibility to penicillin, is not entirely clear but probably depends on their being protected in some way from the action of the antibiotic.

In the present series the persistence of pneumococci could not be clearly related to bacteremia, to underlying respiratory disease, to resistance of the organisms to penicillin, to the numbers present before treatment, or to the failure of the antibody mechanism, at least as judged from the presence of phagocytosis in the sputum smears. The failure to observe such correlations may be due in part to the small number of patients, but it is more likely a result of the relatively great effectiveness of penicillin as an antipneumococcal agent.

It is highly probable that most of the pneumococcus strains which were first recognized after penicillin therapy was started were actually present before that time but in too small numbers to be recognized among the other pneumococci which predominated. They were almost all found during the first day or two of treatment and disappeared quite rapidly.

The changes in the flora of the sputum under penicillin therapy is also of interest. Gram-negative bacilli replaced the more susceptible gram-positive cocci and predominated in most instances after recovery. The complicating staphylococcal pneumonia represents an example of a patient in whom a virulent but penicillin-resistant organism becomes predominant and replaces the original infecting organism. In this patient the staphylococcus was clinically more susceptible to sulfadiazine than to penicillin. It is of interest that in the two patients who died the pneumococci cleared rapidly and death in each case was due to other immediate causes.

SUMMARY

Sputum and throat cultures and stained smears of sputum were studied before, during, and after treatment with penicillin in twenty-six patients with pneumococcal pneumonia. Considerable differences were noted in the persistence of pneumococci in these patients. About one-sixth of the strains found before penicillin therapy was started persisted throughout the period of observation. About three-fourths of the remaining strains could no longer be found after the third day of treatment.

Strains of pneumococci of types not identified in the pretreatment cultures were found for the first time during or after penicillin therapy in six patients. Only one of the strains, however, was found in more than a single specimen.

All of the strains of pneumococci tested were sensitive to penicillin, and three strains isolated after therapy were found to be just as sensitive as the pretreatment strains of the same types isolated from the same patients.

The persistence of pneumococci in the sputum did not appear to be definitely related to bacteremia, complications, chronic respiratory infections, the numbers of organisms present before treatment, the development of penicillin resistance, or the failure to develop antibodies, as judged by phagocytosis of pneumococci in stained smears.

In one patient a staphylococcal pneumonia developed as a complication of the pneumococcal lobar pneumonia. The staphylococcus first appeared in the culture of the sputum obtained twelve hours after penicillin was started and then became predominant in subsequent cultures. It was quite resistant to penicillin, and improvement occurred only after intensive sulfonamide therapy.

Following penicillin therapy, the flora of the sputum changed in most instances to one in which the predominant organisms were gram-negative bacilli having the morphology and colony characteristics of influenza bacilli.

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PRODUCTION OF ANEMIA IN A PIG WHICH RESPONDED TO PURIFIED LIVER EXTRACT

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CASTLE and co-workers¹ demonstrated that several commercial crude casein preparations possessed significant "extrinsic factor" activity and that careful purification was required for the elimination of the extrinsic factor. Borden's Labco vitamin-free casein was found to be essentially free of such activity. Since our swine experiments² were performed with crude casein, it seemed desirable to ascertain the effect of the substitution of Labco vitamin-free casein for the crude Sheffield new process casein used, thus eliminating the extrinsic factor from the diet. The following experiment was performed with this in mind but was carried out only in one animal because of the great cost of the "vitamin-free" casein. The animal was also given sulfasuxidine in the diet. An anemia developed which responded to purified liver extract.

Recently it has been demonstrated in three different laboratories³⁻⁵ that synthetic "folic acid" (*Lactobacillus casei* factor from liver) has antipernicious anemia activity. Because of this fact and its significance in relation to the causation of the nutritional anemias, it was decided to put our observations in a single animal on record at this time. We are well aware that observations in one animal are not sufficient to permit one to draw definite conclusions. We are also aware of the fact that we have not proved that this animal was deficient in "folic acid." However, because the high cost of the experiment prohibits its being repeated in a large series of animals and because of the interest in experimentally induced anemias at the present time, it was felt that it would be worth while to make our results known since they are clear cut and appear to be unique.

METHODS

A pure-bred Duroc pig, 30 days of age, was placed on a basal diet consisting of Borden's Labco vitamin-free casein, 26.1 per cent; sucrose, 57.7 per cent; lard, 11.0 per cent; salt mixture (swine salt mixture No. 3²), 5.2 per cent. Sulfasuxidine* was added to the diet in an amount of 2.0 per cent. In addition, the animal received blebuded oil (1,800 units of vitamin A, 175 units of Vitamin D per gram),† 0.5 Gm. per kilogram of body weight per day. Vitamins‡ were supplied in crystalline form by placing them in capsules and administering them orally. The quantities of crystalline vitamins given were as

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follows (milligrams per kilogram of body weight per day): thiamine hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; p-aminobenzoic acid, 0.50; inositol, 1.20; choline, 10.0. Full details of the experimental methods have been given elsewhere.²

Plasma iron determinations were made by the method of Kitzes, Elvehjem, and Schuette.⁶ The method of Grinstein and Watson⁷ was followed for the determination of erythrocyte protoporphyrin. Serum copper was measured according to the procedure of Cartwright, Jones, and Wintrobe.⁸

RESULTS

After about sixty-five days, thinning of the hair over the rump and back was noted. The animal began to develop an untidy appearance. By about ninety days there was almost complete denudation over the flanks and rump and thinning of hair elsewhere (Fig. 1). Black crusts appeared about the eyelids (Fig. 2). There was a definite impairment of growth (Fig. 3). At 170 days of age the animal weighed 29 kilograms as compared with a normal of 72 kilograms. The gait at all times was not remarkable.

On the one hundred twentieth day of the experiment 1 mg. of biotin* was given intramuscularly and this therapy was continued for seventeen days. There was no change in the general appearance, coat, growth (Fig. 3), or blood (Fig. 4) of the animal. On the one hundred forty-first day of the experiment the volume of packed red cells had fallen to 21 c.c. per 100 c.c. The anemia was normocytic and very slightly, perhaps not significantly, hypochromic. The erythrocyte protoporphyrin (Fig. 4) was in the lower range of normal for pigs. The plasma iron, which had previously been elevated, had fallen to normal. The serum copper was definitely elevated. Unfortunately neither a white cell nor platelet count was done at this time. On the one hundred twentieth day of the experiment, at which time no anemia was present, the white cell count was 17,400 and the platelet count 370,000.

Purified liver extract,† 1 c.c. intramuscularly, was administered on the one hundred forty-first day and this dosage was continued for the next nine days. By the fifth day there was a definite reticulocyte response which reached a maximum of 9.4 per cent on the tenth day of therapy (Fig. 4). There was an immediate rise in hemoglobin and volume of packed red blood cells. The plasma iron after some fluctuation rose to the previous high level. The serum copper slowly fell until it reached the normal range. There was a slight transient rise in the erythrocyte protoporphyrin during the reticulocytosis. Following the initial response there was a gradual increase in the hemoglobin and volume of packed red cells to the lower range of normal and a marked resumption of growth took place. Fifty days after the liver therapy the animal had gained 30 kilograms of weight, a new complete and glossy coat of hair had appeared, and the animal, except for a moderate stunting of growth, was normal.

*Kindly furnished by Merck & Co., Inc., New York, N. Y., through the courtesy of Dr. D. F. Robertson.

†Parke, Davis & Co., 15 units per cubic centimeter.



Fig 1—Marked alopecia, especially in the hind quarters of a pig fed vitamin-free casein and sulfasuxidine.

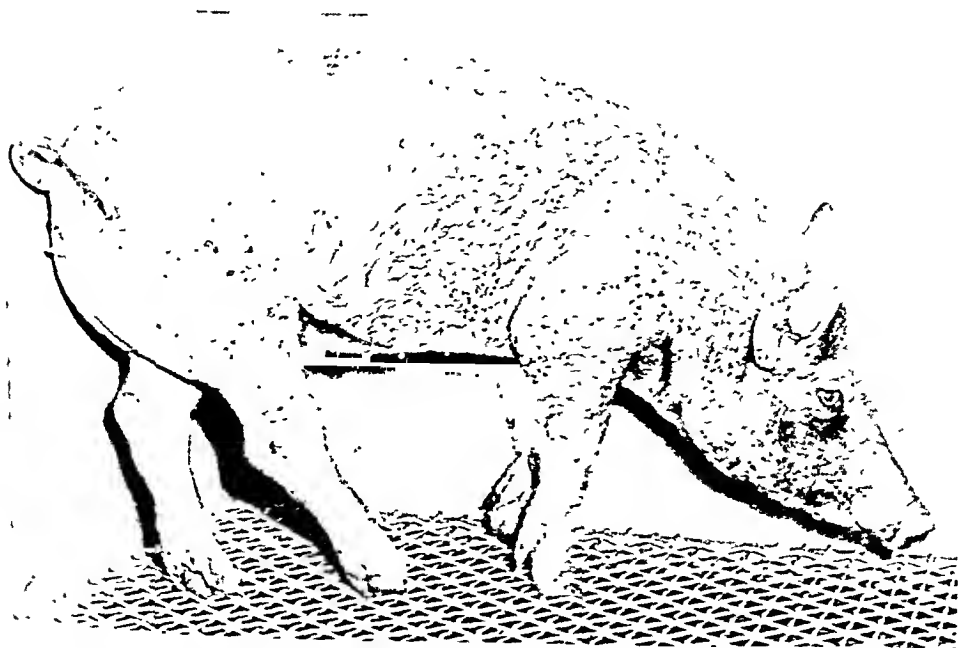


Fig 2—Same animal as shown in Fig 1. Note the black crusts about the eyelids.

To determine whether or not these results could be reproduced by feeding sulfasuxidine in the diet in the presence of crude casein rather than purified casein, four animals were given the same diet as this animal received except that Sheffield's "new process" casein was substituted for Borden's Labco

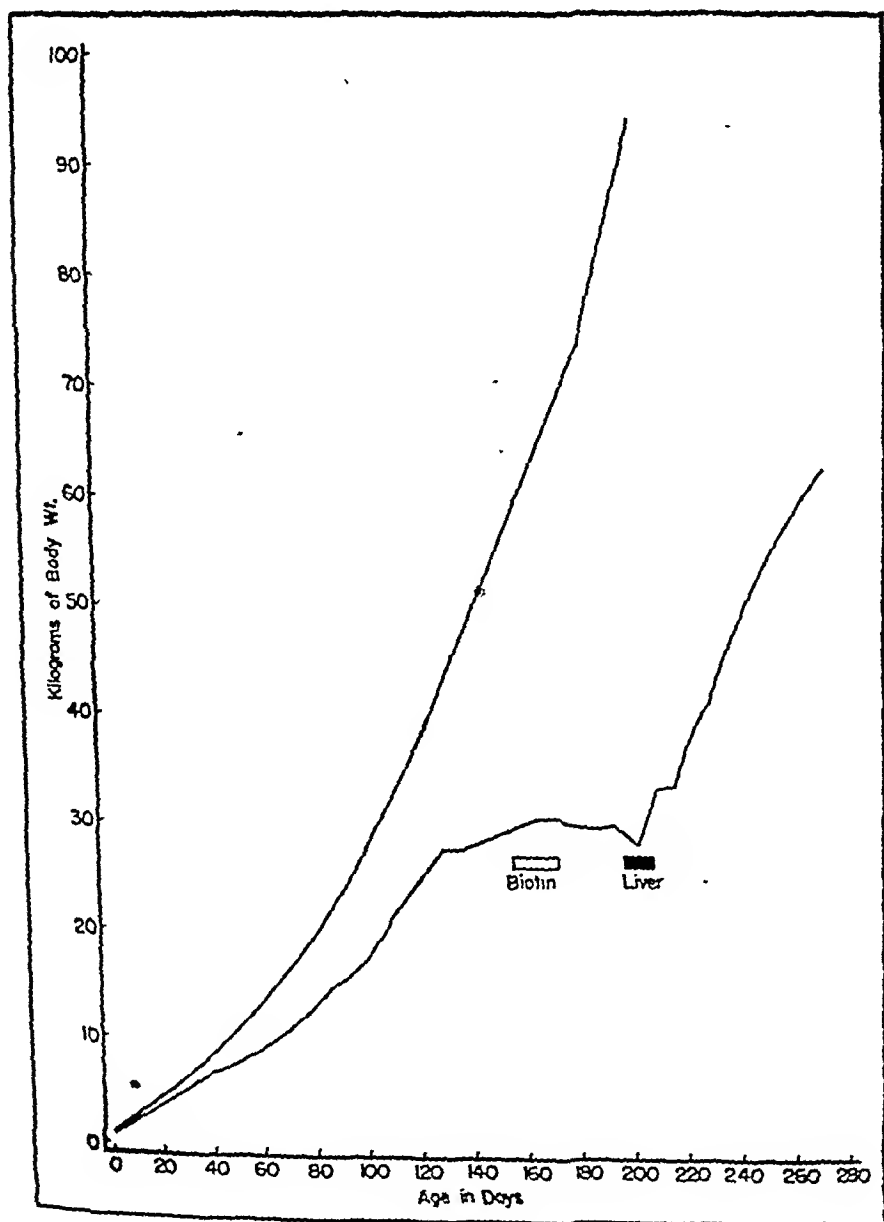


Fig. 3.—Comparison of the growth of the pig shown in Figs. 1 and 2 with that of an animal given crude casein and no sulfasuxidine. Note the failure of biotin therapy and the prompt growth response to the injection of liver extract.

vitamin-free casein. After seven months on this regimen no anemia had developed, the hair was abundant and of good texture, and there was no evident impairment of growth.

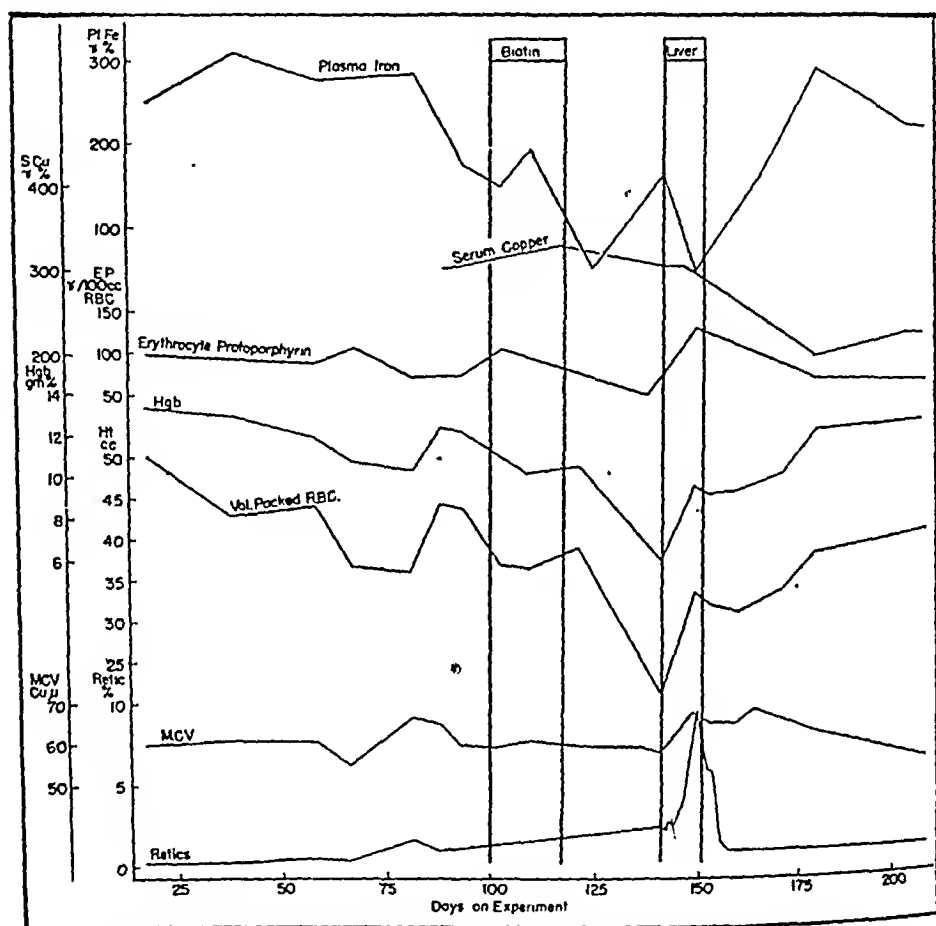


Fig. 4.—Changes in the blood of the animal given crude casein and no sulfasuxidine. Note the reticulocyte response and the relief of anemia following liver therapy.

COMMENT

An anemia developed in this animal which responded to highly purified liver extract. Whether the anemia occurring in this animal was the counterpart of pernicious anemia in human beings we are unable to state. The diet used in this experiment presumably contained no "extrinsic factor." As in pernicious anemia, the serum copper was high and the erythrocyte protoporphyrin normal. Pernicious anemia, however, is macrocytic and is usually accompanied by elevated plasma iron, whereas in this animal the anemia was normocytic and the plasma iron was normal.

In our animal the anemia was produced in the same fashion as "folie acid" deficiency anemia is induced in rats.¹⁰ Borden's Labco casein contains very little "folie acid."¹² Since the administration of "folie acid" (*L. casei*

factor) produces a hemopoietic response in pernicious (macrocytic) anemia in man,³⁻⁵ it is interesting that "folie acid" deficiency in chicks is macrocytic,⁹ whereas in rats¹⁰ and monkeys¹¹ a similar deficiency gives rise to a normocytic anemia. It is possible that in the pig "folie acid" deficiency is associated with the development of normocytic anemia.

SUMMARY

One pig maintained on a diet in which purified casein (Borden's Labco vitamin-free) was substituted for crude casein (Sheffield new process) and to which 2 per cent sulfasuxidine was added failed to grow normally and developed partial alopecia and a normocytic anemia. Following treatment with a highly purified antipernicious anemia liver extract, body hair growth was resumed, and following a definite although not marked reticulocytosis the blood returned to normal. The anemia was accompanied by normal erythrocyte protoporphyrin, normal plasma iron, and an elevated serum copper level.

Four animals maintained on a diet in which crude casein was used in place of the purified casein and to which sulfasuxidine was added grew well, maintained normal coats, and failed to develop anemia.

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THE RELATION OF THE PLASMA SALICYLATE LEVEL TO THE DEGREE OF HYPOPROTHROMBINEMIA

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INTEREST has been renewed in the use of massive doses of sodium salicylate for the treatment of rheumatic fever since the report by Coburn¹ of results with this form of therapy not attained by the use of smaller doses. It has become apparent, however, that large doses of this drug produce hypoprothrombinemia somewhat analogous to that caused by dicumarol. In this study, plasma salicylate levels and prothrombin determinations were performed on a number of patients and rabbits receiving large doses of sodium salicylate. It seemed worth while to determine whether the degree of hypoprothrombinemia might be correlated with the total daily dose of sodium salicylate or with the level of salicylate in the plasma. Elsewhere we have indicated that many clinical manifestations of salicylate intoxication may be correlated closely with the height of the plasma salicylate level which in a given individual may be difficult to predict from the dosage employed.² Actually the salicylate content of the plasma of one individual may be greater than that of another individual who is receiving twice as much of this drug per kilogram of body weight. In previous studies attempts to correlate hypoprothrombinemia with salicylate medication have usually referred to the total salicylate dose rather than to the plasma salicylate level.³⁻⁵ In a few instances, the effect of large doses of synthetic vitamin K was observed in man and animals in our studies.

There is abundant clinical evidence that sodium salicylate and other salicylate derivatives may produce hemorrhage into the gastrointestinal tract and elsewhere. Instances of such complications prior to 1940 have been reviewed by Link and co-workers^{3, 6} and will not be discussed here. More recently additional cases with hemorrhagic manifestations have been observed in individuals with salicylate intoxication.^{7, 8}

Link and co-workers³ have demonstrated the occurrence of hypoprothrombinemia after a single oral or intravenous dose of salicylic acid to rats which were maintained on a diet deficient in vitamin K. If the diet were adequate in vitamin K or supplemented by synthetic vitamin K, hypoprothrombinemia could be prevented. Rapoport, Wing, and Guest⁹ were able to produce hypoprothrombinemia in normal rabbits after a single injection of methyl salicylate.

Many reports are now available which indicate that single or repeated doses of sodium salicylate or other salicylate derivatives, in suitable amounts, result in hypoprothrombinemia in man.^{4, 5, 9-12} Of interest in this regard are

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the observations of Shapiro and co-workers^{4,5} who reported that hypoprothrombinemia usually can be prevented if synthetic vitamin K is administered with the salicylate medication. Most of their patients who were receiving 6 Gm. of acetylsalicylic acid daily developed no hypoprothrombinemia if they also received 6 mg. of vitamin K daily. However, hypoprothrombinemia occurred in one patient on this regime and disappeared after daily intravenous infusions of cevitic acid were given. Meyer and Howard¹² also found that small doses of vitamin K would prevent the hypoprothrombinemic effect of salicylates. Fashena and Walker¹⁰ commented on the efficacy of vitamin K in causing the disappearance of prothrombinopenia in salicylate intoxication.

MATERIAL AND METHODS

The clinical material consisted of cases of acute rheumatic fever and active rheumatoid arthritis treated with large doses of sodium salicylate. These patients received an ordinary hospital ward diet without added vitamins. The prothrombin times and the salicylate levels of the plasma were determined repeatedly in six children and eighteen adults. The sodium salicylate was given intravenously or orally. The range of dosage was from 0.06 to 0.28 of a gram per kilogram of body weight per day in the adults (from 5.4 to 18 Gm.) and was from 0.11 to 0.31 of a gram per kilogram of body weight in the children.

Similar studies of prothrombin times and salicylate levels of the plasma were made in adult rabbits (mostly female) which ranged in weight from 2.2 to 3.4 kilograms. Administration of sodium salicylate was attempted orally, intraperitoneally, intravenously, and subcutaneously. In our hands, the most satisfactory route appeared to be the subcutaneous one, and this was employed exclusively in the animals to be considered. Sodium salicylate in the form of an isotonic solution was given subcutaneously in divided doses at eight-hour intervals for periods ranging from two to thirty days. The total daily dose varied from 0.15 to 1.0 Gm. per kilogram in different animals. As in the case of patients, the rabbits receiving similar doses by identical routes nevertheless showed marked variation in their plasma salicylate levels, even though these were determined each morning eight hours after the last injection and just prior to the next injection in all animals.

Many animals became fatally intoxicated within a short period, while other animals gave no evidence of intoxication. Samples of blood were obtained with difficulty by cardiac puncture from the animals that received salicylates because of the frequency of cardiac tamponade after this procedure. Therefore blood for prothrombin studies and salicylate levels was removed from the femoral veins under direct exposure in nonanesthetized animals. Since so many animals became fatally intoxicated, it was possible to make frequent determinations in only nineteen rabbits.

Following a single injection of sodium salicylate subcutaneously in rabbits, there was a prompt rise in the plasma level to a peak value within thirty minutes. This level then dropped rapidly during the first three hours and was

often too low to measure after four hours. On the other hand, repeated injections produced a cumulative effect which varied considerably in different animals as reflected in the height of the plasma level eight hours after the previous injection.

The plasma prothrombin times were determined by the one-stage method of Quick.¹⁴ This consists in determining the clotting time of recalcified plasma to which an excess of thromboplastin is added. Commercial thromboplastin* was employed. A curve was constructed from the prothrombin times of various concentrations (100, 75, 50, and 25 per cent) of the plasma of a normal person or rabbit. The dilutions were made with physiologic saline. The prothrombin time of the undiluted plasma of the patients or animals receiving salicylates was applied to the curve constructed from the control plasma (rabbit or human), the value of the unknown being expressed as per cent of the normal plasma dilution. A new control curve was prepared each time prothrombin measurements were made. It was not possible to apply human and rabbit prothrombin times interchangeably to the same control curve. The prothrombin times of undiluted and diluted rabbit plasma are much shorter than are those of human controls. Many curves were constructed from the plasma of the same human control and were remarkably constant from day to day. The commercial thromboplastin did not have maximum potency and the slope of the curves in the range 100 to 50 per cent of normal approximated that observed by Souter and Kark,¹⁵ who indicated the advantages of using a relatively weak thromboplastin. With a more potent preparation of thromboplastin, little or no difference in prothrombin time is observed in the 100, 75, and 50 per cent saline dilutions of control plasma.

Link and associates^{6, 16} advocate determining the prothrombin time of a 12.5 per cent saline dilution of the unknown plasma. This method is particularly valuable in detecting slight deviations from normal, since at this dilution a slight variation in prothrombin content alters the time interval considerably. These investigators use a potent preparation of thromboplastin, and, in their hands, the method apparently is reliable even with great reduction in plasma prothrombin content. However, with the available commercial thromboplastin, the normal prothrombin time of a 12.5 per cent saline dilution is often as long as 100 seconds. Marked reduction in prothrombin content greatly lengthens this time, often giving an end point which is difficult to read. Butt and co-workers¹² experienced the same difficulty with the 12.5 per cent dilutions in patients receiving sodium salicylate.

Plasma salicylate levels were determined by the method of Brodie, Udenfriend, and Coburn.¹⁷ Values for plasma salicylate are expressed as micrograms of salicylic acid per cubic centimeter of plasma. Dividing this value by 10 gives the concentration in milligrams per 100 c.c. Menadione was the type of synthetic vitamin K employed. It is dispensed as an aqueous solution in 60 mg. ampules. This was administered intravenously and intramuscularly to human beings and intramuscularly to rabbits.

*Winthrop Chemical Co., Inc., New York, N. Y.

RESULTS

The severity of the hypoprothrombinemia of the plasma of patients receiving sodium salicylate usually was proportional to the level of salicylate in the plasma, increasing with higher plasma salicylate levels (Table I, Figs. 1 and 2). In a given individual, this relationship between plasma salicylate level and degree of hypoprothrombinemia was striking. Different individuals, however, showed some variation in the readiness with which the prothrombin level was reduced in association with a rise in the height of the salicylate level. Usually no significant hypoprothrombinemia (below 40 per cent) occurred until the plasma salicylate level exceeded 600 micrograms per cubic centimeter.

In rabbits, as well as in man, there was a definite correlation between the severity of hypoprothrombinemia and the height of the plasma salicylate level (Table II, Fig. 3). When the salicylate level of the plasma was low, there was no reduction in the prothrombin content although the same dose of sodium salicylate administered to other animals frequently produced high levels and hypoprothrombinemia. One rabbit in this study was given sodium salicylate in the usual dosage (0.9 Gm. per kilogram per twenty-four hours subcutaneously) for a period of thirty days, and yet no reduction in the prothrombin content of the plasma occurred. The plasma salicylate level remained low at all times.

Four patients were given menadione during the course of salicylate therapy. In three individuals, doses as large as 60 mg. daily administered intravenously failed to alter the existent hypoprothrombinemia (Figs. 1 and 2). In one case, 120 mg. of menadione injected intravenously on one day followed by 180 mg. by the same route on the next day restored the prothrombin content to normal for a short period (Fig. 2, Case V. W.). A single intravenous infusion of 500 mg. of cevitamic acid which was given to two individuals with hypoprothrombinemia did not have any effect.

Menadione was administered intramuscularly to three rabbits in doses of 10 mg. daily. In each animal, the vitamin K administration was begun on the day that salicylate therapy was instituted. These animals (Fig. 3) developed high plasma salicylate levels and hypoprothrombinemia occurred while repeated injections of vitamin K were being continued.

In spite of the frequent occurrence of marked prolongation of the prothrombin time of the plasma of patients receiving salicylate therapy, there was evidence of bleeding in only one instance. This patient, a healthy adult, had bleeding from the nose and gums at a time when there was marked hypoprothrombinemia (20 per cent of control curve) and severe salicylate intoxication. In another case, with fatal salicylate intoxication and equally severe hypoprothrombinemia, autopsy revealed only a few insignificant hemorrhages in the serous membranes. In at least five other patients with severe salicylate intoxication and great reduction in prothrombin content of the plasma, no spontaneous hemorrhages were observed.

We have produced fatal intoxication in over 100 rabbits by repeated injections of large doses of sodium salicylate. Gross autopsy examinations, which were made in all animals, usually disclosed a few small hemorrhages in serous

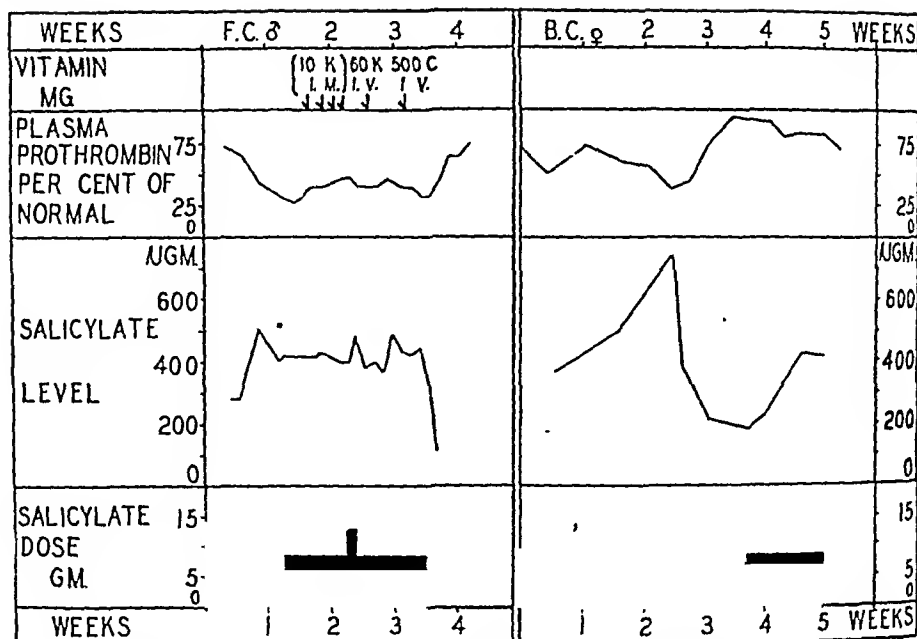


Fig. 1.—The relationship of plasma salicylate level to plasma prothrombin content in two patients. Note the apparent lack of response to vitamin K and vitamin C in the first case.

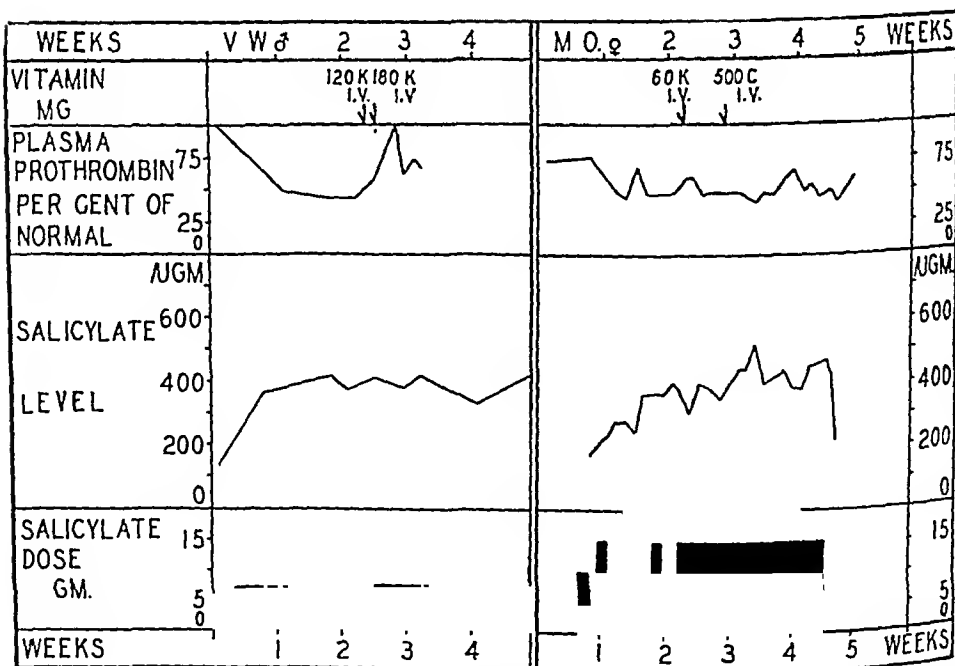


Fig. 2.—The relationship of plasma salicylate level to plasma prothrombin content in two patients. Note the apparent temporary effect of large doses (from 120 to 180 mg.) of vitamin K in the first case and the lack of response to vitamin C and vitamin K in the second case.

TABLE I. RELATIONSHIP OF PLASMA PROTHROMBIN CONTENT TO THE PLASMA SALICYLATE LEVEL IN MAN

	PLASMA PROTHROMBIN CONTENT		
	BELOW 40%	FROM 40-60%	ABOVE 60%
Number of cases	5	14	5
Mean salicylate level ($\mu\text{g./c.c.}$)	644	424	296
Range ($\mu\text{g./c.c.}$)	405-810	264-840	260-350

TABLE II. RELATIONSHIP OF PLASMA PROTHROMBIN CONTENT TO THE PLASMA SALICYLATE LEVEL IN RABBITS

	PLASMA PROTHROMBIN CONTENT		
	BELOW 40%	FROM 40-75%	ABOVE 75%
Number of cases	6	9	4
Mean salicylate level ($\mu\text{g./c.c.}$)	477	337	140
Range ($\mu\text{g./c.c.}$)	300-640	90-568	20-280

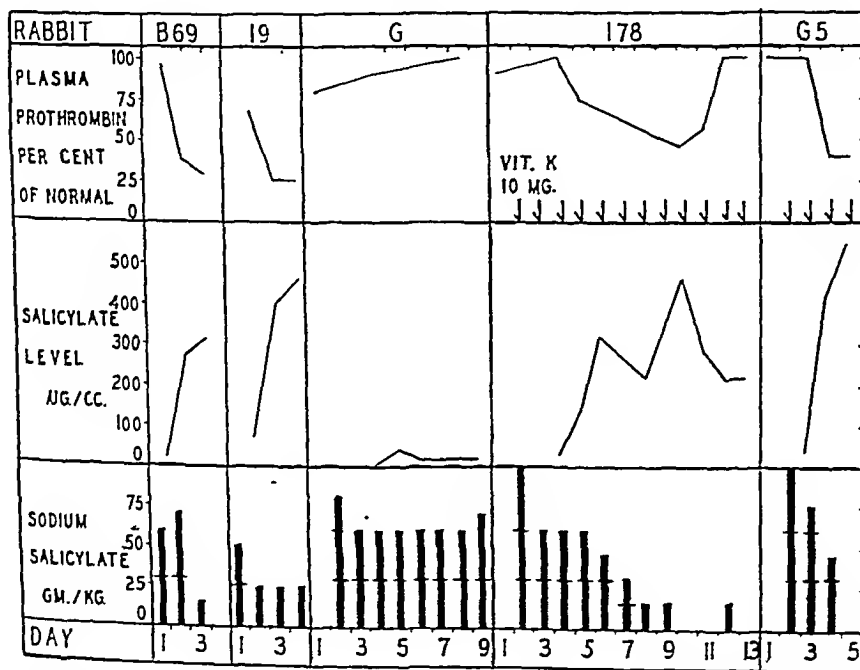


Fig. 3.—The relationship of plasma prothrombin content to plasma salicylate level in five rabbits. Note the apparent failure of vitamin K to prevent hypoprothrombinemia in the last two animals.

membranes but never any severe hemorrhage which might have been responsible for the death of the animals (the central nervous system was not examined).

Some in vitro studies were made to determine the influence of sodium salicylate upon the prothrombin content of the plasma. The concentration of sodium salicylate (added as dry powder) varied from 250 to 1,000 micrograms per cubic centimeter in various experiments. Prothrombin times were determined immediately; after twenty-four hours' incubation at 37°C. ; after forty-eight and ninety-six hours at 4°C. ; and after 120 hours at room temperature.

using sterile technique. At the temperature of 4° C., the prothrombin content of both the control plasmas and the plasmas to which salicylates had been added were little affected, even after from two to four days' standing. With the higher temperatures, a progressive reduction of prothrombin content of equal degree occurred in both control plasmas and in those containing salicylates. This action was more pronounced at 37° C., particularly after prolonged incubation. A similar destruction of prothrombin in normal plasmas permitted to stand at room temperature has been reported by Taylor and co-workers.¹⁴ The failure to demonstrate an *in vitro* action of salicylate in concentrations attainable *in vivo* has also been observed by Link and co-workers.³

COMMENT

These studies indicate that the severity of hypoprothrombinemia in patients and experimental animals may be correlated with the height of the plasma salicylate level, the degree of hypoprothrombinemia increasing with high salicylate levels. The plasma salicylate levels in a given individual may not be predicted from the dose employed since there is great individual variation in the dosage necessary to obtain comparable plasma levels in different persons.

Butt and co-workers¹² likewise noted the hypoprothrombinemia associated with salicylate medication in patients with rheumatic fever but could find no correlation between plasma salicylate level and degree of reduction in plasma prothrombin. They expressed the opinion that the likelihood of development of severe hypoprothrombinemia and spontaneous hemorrhage in association with salicylate medication was remote. We are in accord with this opinion. Recently in a preliminary report, Coombs, Higley, and Warren¹³ observed that the hypoprothrombinemia occurring with salicylate therapy is in direct proportion to the plasma salicylate level and that the severity of hypoprothrombinemia is of minor degree until the plasma salicylate level reaches 600 micrograms per cubic centimeter. This confirms our observations.

Previous reports have emphasized the value of vitamin K in preventing the development of hypoprothrombinemia as well as in hastening the restoration of the prothrombin content when salicylates have been discontinued.^{4, 5, 13} In the present work, limited observations in rabbits indicate that when the plasma salicylate level is high, hypoprothrombinemia may occur in spite of continued administration of vitamin K. In man, we did not begin vitamin K medication at the time of initiating salicylate therapy; however, moderately large doses of vitamin K (from 60 to 120 mg. intravenously) after the hypoprothrombinemia had developed had little or no effect while salicylate therapy was being continued.

The mode of action of salicylates upon the prothrombin content of the plasma would seem to be via the liver. It is a well-established fact that vitamin K is necessary for synthesis of prothrombin by the liver. Animals fed diets low in vitamin K³ and patients with cirrhosis of the liver⁵ have been found to be more susceptible to the hypoprothrombinemic effect of salicylates. In the patients and rabbits studied in the present work, there was no reason

to suspect a dietary lack of vitamin K or the presence of antecedent damage to the liver.

Since salicylates, dicumarol, and vitamin K contain salicyl groups in their molecular structures, it seems possible that, although sufficient vitamin K is present, its utilization may be blocked by salicylates or by dicumarol. Massive doses of vitamin K may overcome this interference, perhaps by a mass action effect. The hypoprothrombinemia produced by dicumarol in patients usually may be overcome by the administration of very large doses of vitamin K.^{20, 21}

The lowering of the prothrombin content may be the result of the influence of salicylates on certain enzyme systems. Serious liver impairment does not appear to be a frequent finding during salicylate medication, especially as evidenced by the usual liver function tests.¹² However, ketonuria may occur if salicylate intoxication is severe. Moreover, in vitro studies have demonstrated alterations of certain metabolic activities of tissue slices in the presence of salicylates.²²⁻²⁵ The rapid restoration of the prothrombin content of the plasma after the discontinuance of salicylate therapy does not suggest that any permanent liver damage occurs.

SUMMARY

A correlation between the height of the level of salicylate in the plasma and the degree of hypoprothrombinemia was established as a result of studies on twenty-four patients and nineteen rabbits receiving large doses of sodium salicylate.

Moderately large doses of vitamin K administered to rabbits throughout the period of salicylate medication and to man during the course of salicylate medication did not appear to have any effect in regard to the development or persistence of hypoprothrombinemia while salicylate therapy was continued.

The prolonged prothrombin time does not appear to be of clinical significance since hemorrhagic phenomena in intoxicated animals and man are infrequent and, when present, usually do not appear to be a factor in causing death from salicylate intoxication.

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A CRITICAL ANALYSIS OF THE VALUE OF THE ADDITION OF A AND B GROUP-SPECIFIC SUBSTANCES TO GROUP O BLOOD FOR USE AS UNIVERSAL DONOR BLOOD

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WITH THE TECHNICAL ASSISTANCE OF TECHNICAL SERGEANT JAMES COSCIA
AND STAFF SERGEANT JOHN GLACH

IN A RECENT paper¹ it was demonstrated that transfusions of group O blood with an isoagglutinin titer of 400 units or higher (by the tube-centrifuge method of titration) to recipients of other blood groups resulted in hemolytic reactions of varying severity. On the basis of this work, only group O blood with an isoagglutinin titer of 200 units or less was recommended as safe for routine use as universal donor blood. Many investigations have been carried out (Witebsky and associates,² Klendshoj and co-workers,³ and Wiener and associates⁴) on the use of soluble A and B group-specific substances for addition to group O blood to reduce its isoagglutinin titers to safe levels. The ability of these substances to reduce the titer of anti-A and anti-B agglutinins in vitro has been established. Klendshoj and associates³ have reported clinical studies in a large series of patients on the effect of transfusions of *random* group O bloods to which solutions of group substances had been added. However, no studies have been made of the effects of the transfusion of *selected* group O bloods known to have a high titer of isoagglutinins which had been treated with A and B substances and then given to recipients of other blood groups.

Accordingly, this investigation was undertaken with the following problems in mind: (1) to determine the amount of blood, with a high titer of isoagglutinins, necessary to cause a hemolytic reaction in a recipient of a different blood group; (2) to study the in vitro effects of group-specific substances A and B on plasma with a high isoagglutinin titer*; (3) to determine the effects of the transfusion of blood with a high isoagglutinin titer, after modification with A and B group substances, to recipients of incompatible blood groups.

MATERIALS AND METHODS

For this investigation, blood plasma was used instead of whole blood because the antibodies, whose effects were being studied, are present only in the plasma, so that one could be sure that any hemolysis which occurred was due to destruction of the red cells of the recipient.

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*The group-specific substances were generously supplied by Dr. E. J. Tectter, of Eli Lilly and Co., Indianapolis, Ind.

Group B plasma, having a very high titer of anti-A agglutinins, was obtained* from a blood donor who had been immunized by an injection of soluble group-specific substances. The titer of the anti-A agglutinins for A_2 cells, by the well-slide method, reached as high as 2,500 units (equivalent to 15,000 units by the tube-centrifuge technique) but had fallen to a titer of 500 units (2,500 units by the tube technique) by the time the plasma was collected from the donor some three months after the injection of the group substances. One thousand cubic centimeters of this plasma were obtained at one time by withdrawing 1,800 c.c. of blood from the donor and simultaneously replacing it with an equal volume of group B bank blood.

This plasma, both in the untreated form and with A and B group substances added in the proportion of 10 c.c. of W. Tebsky's solution to 250 c.c. of plasma, was given in varying doses to eight volunteer recipients recruited through the cooperation of the Colorado State Penitentiary.† Twelve 250 c.c. bottles of group O plasma with high anti-A agglutinin titers‡ were administered, after neutralization with 10 c.c. of A and B substances, to twelve other volunteers.

Each volunteer recipient was given a thorough physical examination. Only healthy men were used. The recipient's blood group was determined and he was classified as a secretor or a nonsecretor, according to the technique of Wiener.⁵ Volunteers of subgroups A_1 and A_2 alone were transfused. The plasma was titrated for its isoagglutinin content against the red cells of the prospective recipient. Except where otherwise indicated, all titrations were performed by the tube-centrifuge technique. In those cases in which the A and B soluble substances were added, titrations against the recipient's red cells were performed before and after neutralization. Twenty-four hours prior to each transfusion, the hemoglobin concentration (using the Fisher hemometer), red blood cell count, hematocrit, serum bilirubin, and urine urobilinogen values of each recipient were ascertained.§ These tests were repeated one hour after the end of the transfusion and every twenty-four hours thereafter for seven days. Immediately at the termination of the infusion, the recipient's blood was examined for evidence of spontaneous agglutination. The technique for determining this phenomenon and its significance have been discussed previously.¹ All recipients were alkalized with sodium bicarbonate before the plasma was given.

RESULTS

Our findings are summarized in Table I. The data for the third, fifth, and sixth days are not given for the sake of brevity and because they showed no significant deviation from the days shown.

As noted in Table I, as little as 25 c.c. of the untreated group B plasma administered to a group A_1 recipient (Case 1) caused a frank hemolytic reaction, as manifested by chill, fever, pain in the lumbar region, hemoglobinuria,

*We gratefully acknowledge the aid given us by Dr. Lester J. Unger, of the New York Post-Graduate Hospital Blood Bank.

†We desire to express our deep appreciation for the cooperation of Warden Roy Best and the volunteers of the Colorado State Penitentiary, Canon City, Colo.

‡Furnished to us by the Department of Surgical Physiology at the Army Medical School, Washington, D. C., through the courtesy of Major John McGraw, Medical Corps, Army of the United States.

§Colonel Hugh S. Mahon, Medical Corps, United States Army, Chief of Laboratory Service, Fitzsimons General Hospital, Denver, Colo., generously provided the services of his laboratory.

and increased bilirubinemia, together with evidence of spontaneous agglutination. Fifty cubic centimeters caused an even more pronounced reaction in another group A₁ recipient (Case 2). In a volunteer of group A₂ (Case 6), 25 c.c. of this plasma caused no obvious reaction, but 50 c.c. (Case 7) gave rise to convincing clinical evidence of hemolysis of the recipient's cells. This confirms our previous work on this subject and demonstrates the small quantity of blood with a high isoagglutinin content that is sufficient to cause a hemolytic reaction if given to recipients of an incompatible blood group. On the other hand, when as much as 250 c.c. of this group B plasma were administered, after addition of 10 c.c. of the Witebsky group substances, to two volunteers (one group A₁, the other A₂), no manifest signs of hemolysis resulted. In one instance only was evidence of spontaneous agglutination observed, but this patient (Case 5) showed no other convincing findings indicating hemolysis.

Twelve additional A₁ recipients were then given transfusions of the group O plasmas containing a high titer of anti-A agglutinins which had been modified by the addition of A and B group specific-substances in the proportion of 10 c.c. of the group substances to 250 c.c. of plasma. As shown in Table I, the ability of these substances to reduce the *in vitro* isoagglutinin titers to low levels is established beyond question. The highest titer among the plasmas used was 4,800 units by the tube method. The titer of this plasma was reduced to 100 units by the group substances. In one case in which the original titer was 500 units the presence of anti-A agglutinins could no longer be detected after the addition of the group-specific substances.

No clinical reaction was noted among the individuals receiving "neutralized" plasma except for one case of urticaria which has no bearing on this investigation. No post-transfusion hemoglobinuria was observed, and in only six of the twelve recipients was there a significant increase in bilirubinemia. In the other six recipients, while no marked rise in bilirubinemia occurred, a slight fall in the hemoglobin and red blood cell and hematocrit values suggested a small amount of red cell destruction. However, this could also be explained by the hemodilution resulting from the plasma infusion. In five of the recipients, signs of hemolysis were somewhat delayed, appearing as late as the fourth and fifth post-transfusion day. This might be explained by postulating that the combination of agglutinins and the group substances was a loose one, with a gradual release of the agglutinins. Nevertheless, it is felt that in all these recipients the actual amount of red cell destruction was clinically insignificant. This is predicated on the close observation of the recipients, on the fact that no clinical reactions occurred, and on the fact that in all recipients the evidence of hemolysis was transitory and because the small number of recipient's red cells destroyed probably did not exceed the equivalent of from 25 to 50 c.c. of blood. Therefore, it may be stated that this study has demonstrated the ability of A and B group-specific substances to inhibit the hemolytic action of the transfusion of group O blood with a high agglutinin titer to recipients of other blood groups.

As has already been mentioned, every recipient's saliva was tested for the presence or absence of group-specific substance A, and each patient was classified

CASE*	RECIPIENT'S BLOOD GROUP	SALIVA TEST	AMOUNT TRANSFUSED (C.C.)	AGGLUTININ TITER OF PLASMA AGAINST RECIPIENT'S CELLS before ADDITION OF A AND B SUBSTANCE	AGGLUTININ TITER OF PLASMA AGAINST RECIPIENT'S CELLS after ADDITION OF A AND B SUBSTANCE	TWENTY-FOUR HOURS BEFORE TRANSFUSION					ONE HOUR AFTER TRANSFUSION					TWENTY- AFTER T		
						HEMOGLOBIN (GM.)	R.B.C.	HEMATOCRIT	BILIRUBIN (MG./100 C.C.)	UROBILINOGEN	SPONTANEOUS AGGLUT.	HB. (GM.)	R.B.C.	HEMATOCRIT	BILIRUBIN (MG./100 C.C.)	UROBILINOGEN	HEMOGLOBIN (GM.)	R.B.C.
1†	A ₁	Sec.	25	1-2400		15	4,680	44.5	1.10	Neg.	Pos.	15.4	4,850	46	2.10	Neg.	14	4,800
2†	A ₁	Sec.	50	1-2400		17	5,810	47.5	0.60	Neg.	Pos.	16	5,600	46.5	2.38	Neg.	16	5,570
3	A ₁	Sec.	25	1-2400	1-40	16	5,950	47.5	0.42	Neg.	Neg.	15	5,300	48	1.00	Neg.	14.7	5,730
4	A ₁	Sec.	50	1-4800	1-100	14.8	5,500	47.5	0.38	Neg.	Neg.	15	5,250	47	1.18	Neg.	15	5,190
5	A ₁	Sec.	250	1-4000	1-32	15	5,470	48	0.59	Neg.	Pos.	14.2	4,690	42	0.82	Neg.	14	4,850
6†	A ₂	Sec.	25	1-2000		14	5,290	46	0.80	Neg.	Pos.	13.3	4,960	41.5	1.20	Neg.	14.8	4,590
7†	A ₂	Nonsec.	50	1-1500		14.8	5,490	44	0.87	Neg.	Pos.	14.8	5,650	45	1.40	Neg.	14.2	5,810
8	A ₂	Nonsec.	250	1-1000	1-4	13.8	4,980	45	0.40	Neg.	Neg.	13.5	4,660	42	0.87	Neg.	13.8	4,820
9	A ₁	Nonsec.	250	1-1200	1-24	16	5,400	45	0.50	Neg.	Neg.	13.5	5,170	42	1.10	Neg.	13.5	4,800
10	A ₁	Sec.	250	1-800	1-12	16	5,600	47.5	1.05	Neg.	Neg.	16	5,340	42	0.70	Neg.	15.5	5,250
11	A ₁	Sec.	250	1-1200	1-8	16.5	6,010	51.5	0.90	Neg.	Neg.	15.8	5,650	48	0.50	Neg.	15.8	5,500
12	A ₁	Sec.	250	1-1000	1-8	13.9	4,750	43	0.30	Neg.	Neg.	15.8	4,750	44.5	0.90	Neg.	16	5,310
13	A ₁	Sec.	250	1-600	1-4	14.4	5,240	46	0.46	Neg.	Neg.	12.5	4,810	43	0.65	Neg.	14.2	5,980
14	A ₁	Sec.	250	1-1000	1-8	15.5	5,850	50	0.80	Neg.	Neg.	16	5,450	45	0.87	Neg.	13.6	5,820
15	A ₁	Sec.	250	1-1000	1-4	15	5,800	46	0.70	Neg.	Neg.	14.2	5,530	44	0.87	Neg.	16	5,490
16	A ₁	Nonsec.	250	1-1000	1-8	14.6	5,340	45.5	0.45	Neg.	Neg.	14	4,900	42	0.59	Neg.	14	5,280
17	A ₁	Sec.	250	1-2000	1-8	15.4	5,920	44.5	0.40	Neg.	Neg.	12.8	4,860	40	0.59	Neg.	13.2	4,960
18	A ₁	Sec.	250	1-1000	1-4	13.8	5,180	43	0.70	Neg.	Neg.	14.2	4,950	43	1.07	Neg.	15.5	5,270
19	A ₁	Sec.	250	1-1500	1-4	15.2	6,100	46	0.59	Neg.	Neg.	14.4	5,590	43	0.70	Neg.	16	5,630
20	A ₁	Sec.	250	1-500	None detected	15.4	5,620	47.5	0.30	Neg.	Neg.	15	5,370	44.5	0.40	Neg.	15	5,490

*In Cases 1 to 8, the recipients received group B plasma obtained from one special donor. In 9 to 20 the recipients received Group O plasma obtained from individual donors.

†Received unmodified plasma.

as a secretor or a nonsecretor. Whether the recipients were secretors or non-secretors had no obvious effect on the results of the transfusion experiments.

During the course of this project, determinations were made of the titer of the anti-B agglutinins in the sera of those group A recipients who had received group O plasma neutralized with A and B substances. The titrations were performed before the transfusion and at weekly intervals thereafter. Only those recipients who had received modified group O plasma were studied because group O plasma itself does not contain A or B group substances. Therefore, any antibody stimulation in those recipients would be due solely to the action of the added A and B group-specific substances. It has been shown previously that such substances do stimulate antibody formation and indeed their injection has been used to produce potent blood grouping serum of a

TEST TION	SECOND DAY AFTER TRANSFUSION					FOURTH DAY AFTER TRANSFUSION					SEVENTH DAY AFTER TRANSFUSION					CLINICAL REACTION
	UROBILINOGEN	HEMOGLOBIN (gm.)	R.B.C.	HEMATOCRIT	BILIRUBIN (mg./100 c.c.)	UROBILINOGEN	HEMOGLOBIN (gm.)	R.B.C.	HEMATOCRIT	BILIRUBIN (mg./100 c.c.)	UROBILINOGEN	HEMOGLOBIN (gm.)	R.B.C.	HEMATOCRIT	BILIRUBIN (mg./100 c.c.)	UROBILINOGEN
Neg.	16.2	4,800	45	0.78	Neg.	14.5	4,870	44.5	0.80	Trace	15	4,900	45	0.45	Trace	Temperature, 99.4° F.; chill; lumbar pains; hemoglobinuria
Neg.	16.5	5,500	50	0.86	Neg.	15.5	5,790	45	0.88	Trace	16.4	5,830	48	0.70	Neg.	Lumbar pain; hemoglobinuria
Neg.	14	5,490	46	0.50	Neg.	14.2	6,130	46.5	0.30	Neg.	15.5	5,990	44	0.35	Neg.	None
Neg.	16	5,530	47	0.70	Neg.	13.8	5,590	46	0.30	Neg.	15	5,710	43.5	0.15	Neg.	None
Pos.	14.2	4,710	39	0.75	Trace	15.5	5,250	46	0.72	Neg.	14.6	5,430	43	0.65	Neg.	None
Trace	14	5,100	47	0.90	Pos.	13.8	5,020	43	0.65	Neg.	14	5,480	42	0.72	Neg.	Chill; temperature, 100° F.
Neg.	13.8	5,690	45	0.70	Neg.	15.8	5,420	45.5	0.72	Neg.	15	5,290	40	0.90	Neg.	Hemoglobinuria; chill
Trace	15.5	4,990	47	0.50	Trace	15.8	4,690	44.5	0.70	Trace	14.8	5,060	41.5	0.20	Neg.	None
Neg.	14	5,150	43.5	0.30	Neg.	13.8	5,160	42	0.70	Neg.	13	4,910	45	0.59	Neg.	Urticaria
Neg.	15	5,550	43.5	0.82	Neg.	15.3	5,420	45	1.60	Neg.	15	5,270	46	1.55	Neg.	None
Neg.	14.2	5,450	48.5	0.78	Neg.	15	5,060	46.5	0.30	Trace	15.8	5,890	45.5	1.05	Neg.	None
Neg.	13	4,290	39.5	0.72	Neg.	13.6	4,730	41.5	0.07	Neg.	13.2	4,420	38	0.25	Neg.	None
Neg.	14.8	5,070	43.5	0.83	Neg.	13.2	4,570	41	0.92	Neg.	13.2	5,220	42	0.54	Neg.	None
Neg.	13.5	5,410	47	0.78	Neg.	14.3	5,710	47	0.87	Trace	14.8	5,810	47	0.62	Neg.	None
Neg.	16	5,700	48	0.70	Neg.	15	5,770	47.5	0.42	Neg.	15.2	5,410	49	0.68	Neg.	None
Neg.	15	5,740	46	0.80	Neg.	13.5	5,080	41	1.30	Neg.	15	5,530	45	0.30	Neg.	None
Neg.	15.4	5,350	42	0.35	Neg.	14	5,410	44.5	1.05	Neg.	15.5	5,810	44.5	0.90	Neg.	None
Neg.	15	5,190	45	0.85	Neg.	15.2	4,890	40.5	1.35	Pos.	15.4	5,260	40.5	0.40	Neg.	None
Trace	15.2	5,480	46.5	0.85	Neg.	16.5	5,670	46	0.70	Neg.	15.2	5,590	45	0.87	Pos.	None
Neg.	14	5,640	45	1.05	Pos.	14	5,510	45.5	0.80	Pos.	15.3	5,880	47	0.40	Trace	None

high isoagglutinin content.^{6,7} The results of these titrations are presented in Table II.

It is evident that a definite rise in the anti-B agglutinin titer occurred in all twelve recipients. On the average, there was a sixfold increase in the titers. This, in itself, presents no problem in male recipients. However, in a recent report, Wiener⁸ has described a case of erythroblastosis fetalis caused by the action of the anti-A isoagglutinins of a group O mother on the group A red cells of a fetus in utero. Such an occurrence is more likely when there is a high titer of isoagglutinins in the maternal circulation. It follows, therefore, that there is a slight but definite danger in stimulating increased isoantibody formation in female patients potentially capable of childbearing. On this basis it may be wise to be more discriminating about transfusing such women with group O

TABLE II. TITRATIONS OF ANTI-B AGGLUTININS IN GROUP A RECIPIENTS OF NEUTRALIZED GROUP O PLASMA

CASE	BEFORE TRANSFUSION	FIRST WEEK	SECOND WEEK	THIRD WEEK	FOURTH WEEK
9	1-10	1-20	1-30	1-30	1-40
10	1-10	1-20	1-40	1-80	1-100
11	1-40	1-80	1-120	1-140	1-180
12	1-40	1-80	1-80	1-80	1-100
13	1-30	1-40	1-60	1-60	1-80
14	1-10	1-40	1-80	1-100	1-140
15	1-20	1-40	1-60	1-80	1-100
16	1-10	1-20	1-40	1-80	1-100
17	1-10	1-20	1-40	1-80	1-80
18	1-120	1-150	1-150	1-180	1-300
19	1-100	1-150	1-180	1-200	1-300
20	1-40	1-40	1-100	1-120	1-180

blood modified by group-specific substances. Certainly, however, in the face of an emergency, there should be no hesitancy in using such blood.

COMMENT

There are two fields in medicine where the use of universal donor group O blood plays an important role. It is of prime importance in military medicine in the treatment of combat casualties, where its efficacy has been proved beyond doubt. It has been demonstrated that group O blood must have a low titer of isoagglutinins (about 200 units by the tube technique) to be safe for use as universal donor blood.¹ Such a low titer will mean discarding about 25 to 50 per cent of group O bloods to be used for this purpose. In the presence of a large demand for universal donor blood, such a waste of material would be unwarranted. Based on the observations made in the present study, it is felt that the routine addition of A and B substances to group O blood would render any group O blood perfectly safe for use as universal donor blood for military purposes.

The other important therapeutic role of universal donor blood is its use for the immediate treatment of emergency cases of hemorrhage in civilian life. Low titer group O blood should be transfused while proper groupings and cross-matching tests are being carried out. As an additional precaution, Rh-negative blood should be used. Further observation and follow-up studies must be made on the subject of the possible significance and effects of isoantibody stimulation in women potentially capable of childbearing. In the meantime, because of the impracticability of having two kinds of universal donor blood, one for men and one for women, we believe that low-titer group O blood destined for use as universal donor blood in civilian hospitals should be selected according to the technique outlined in our previous paper,¹ without the addition of A and B substances.

CONCLUSIONS

1. As little as 25 c.c. of a group B plasma containing a high titer of isoagglutinins caused a hemolytic reaction when administered to a group A recipient.

2. A and B group-specific substances will reduce a high isoagglutinin titer to low levels.

3. Experiments on the administration of plasma with high titers of anti-A agglutinins to group A recipients present definite evidence that the addition of group-specific substances A and B is a safe and reliable method for preparing all group O blood for use as universal donor blood for military purposes.

4. A and B substances are capable of antibody stimulation even when in combination with the anti-A and anti-B agglutinins of group O blood. This calls for some caution in administering such material to women potentially capable of childbearing.

5. For use as universal donor blood in civilian hospitals, Rh-negative group O blood, selected for its low natural isoagglutinin titer, is somewhat preferable to random group O blood to which group substances have been added to reduce the isoagglutinin titer.

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UREA AS AN ADJUNCT TO SULFONAMIDE THERAPY

REPORT OF ITS SUCCESSFUL USE IN A CASE OF SUBACUTE BACTERIAL ENDOCARDITIS

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THE renal complications from the use of sulfadiazine are well known and have been adequately described in recent medical literature. When the drug is given in massive doses the possibility of obstructive and toxic damage to the kidney is greater. The administration of alkali has been helpful in preventing or reducing these untoward effects. Urea, too, can be used for this purpose and, in addition, may add to the antibacterial action of the sulfonamide.

Urea or carbamide is a white, colorless, odorless, crystalline substance, highly diffusible, dissolving readily in water, 1 Gm. in 1.1 c.c.¹ Aqueous solutions are neutral and, on heating, break down with the liberation of ammonia and carbon dioxide and, therefore, are not to be sterilized by heating. Urea is marketed in chemically pure form and also as urea reagent,¹ which contains impurities. Obviously the former and not the latter is used for intravenous therapy. On local application it is nontoxic, is fairly innocuous to human tissues, and can be sprinkled directly on wounds.²

Long ago (1902) Ramsden³ showed that it prevented putrefaction and in a dilute solution as 2 per cent had a marked effect in promoting digestion by proteolytic enzymes. Foulger and Foshay² showed that some enzymatic actions may be retarded even by low concentrations.

The first detailed study of urea as a bactericide was made by Peju and Rajat in 1906.⁴ In the same year, Wilson⁵ noted that growth of *Escherichia coli* could be prevented by 8 per cent urea—from 1.5 to 3.5 per cent caused pleomorphism of these organisms.

Urea was used for its direct action in World War I by Symmers and Kirk⁶ to débride contaminated war wounds chemically. They applied (100 per cent) aqueous solutions and also used the solid substance. Kirk had a favorable experience with its clinical use over a period of four years. Holder and Maekay⁷ in 1937 found it of value in the treatment of infected wounds. In 1942 these authors⁸ demonstrated its use together with sulfanilamide in the treatment of infected wounds. They believed the carbamide (urea) action rids the wound of necrotic tissue and similarly of sulfonamide inhibitors, allowing the sulfonamide to exert a maximum bacteriostatic action. They also stated that urea markedly enhanced the solubility of sulfonamides and thus increased their action. Their experience covered a five-year experimental period. This type of inactivation of sulfonamide inhibitors was also described with the use of azochloramide⁹ and urethane.¹⁰

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During 1942 studies in the therapeutic application of urea in infections were reported by Tsuchiya, Tenenberg, Clark and Strakosch.¹¹ They found that urea (1) was nontoxic, a strong peptizing agent with marked solvent action for necrotic tissue, pus, and debris; (2) chemically debrided contaminated wounds and mechanically removed inhibitors; (3) lysed bacteria, deodorized foul-smelling wounds, and rendered sulfonamides more soluble. They particularly showed urea to neutralize methionine and remove sulfonamide fastness. The bacteria used were *Esch. coli* and a staphylococcus which was obtained from a human abscess and made "fast."

Curtis and Sobin, Sobin, and Sobin, Aronberg, and Rolneck¹² (1941-1943) called attention to the sulfonamide-solvent effect of urea and showed that urea might prevent the formation of renal calculi by sodium acetyl sulfapyridine in rats.

Thus far the work had been on the action of urea locally in wounds, and usually in high concentration, in vitro, and in the kidney of rats.

In 1943, when we contemplated treating a patient with subacute bacterial endocarditis with massive doses of sulfadiazine, it was suggested to us by Dick¹³ that urea be used with it. The following is a report on a patient with subacute bacterial endocarditis in whom this method of the use of the sulfadiazine and urea was employed and in whom recovery promptly followed.*

REPORT OF CASE

History.—R. K., a native-born white girl aged 18 years, single and attending trade school, was admitted to the medical service of Beth Israel Hospital Jan. 6, 1943. She was an only child and was told she was born with a weak heart but was not a blue baby. Since the age of 6 years she attended a cardiac clinic about twice a year. During her childhood she played games such as ball and jumping rope without undue difficulty. Her physical activity was not limited until three years before admission to the hospital and then only on the advice of a physician. There was no dyspnea, edema, or palpitation.

About two months prior to admission to the hospital she began to cough, lose weight, and feel ill. This continued for a few weeks, at which time she was seen by a physician who told her she had fever and should remain in bed. Anorexia developed and her temperature rose to 101° F. After several days in bed she felt better and returned to school.

One day before admission to the hospital she became more feverish, visited her physician again, and was advised to seek hospitalization. She had lost thirteen pounds during the two months' illness, felt tired and weak all the time, and had missed her last two menstrual periods.

Examination.—The patient was 5 feet, 3 inches tall, weighed 122 pounds, sthenic habits, appeared acutely ill and quite pale. She was coughing and complained of some pain over the left lower chest and left upper abdomen, aggravated by deep breathing and by coughing. The temperature was 103.2° F.; pulse rate, 102; and respiratory rate, 24. There was slight dyspnea and moderate cyanosis of the nail beds. Petechiae were not found in the conjunctivae or fundi nor on the body or extremities.

The pharynx was slightly congested; teeth were in good repair; the cervical lymph nodes were not palpable. The main features of interest were found on examination of the heart and lungs.

*In the September, 1944, issue of the JOURNAL, Schnitker and Lenhoff¹⁴ reported the use of urea and sulfonamide by mouth in the treatment of sulfonamide-resistant gonorrhea.

In the Feb. 10, 1945, issue of the LANCET Ecker¹⁵ reported the use of urea and sulfadiazine in the treatment of a patient with *B. coli* meningitis.

The heart was not enlarged to percussion; the apical impulse was of moderate force and within the midclavicular line. The first sound at the apex was of normal quality and intensity. There was a loud rough blowing systolic murmur heard over the whole precordium, maximum at the third intercostal space and fourth rib just left of the sternum. The murmur was transmitted to the vessels of the neck and to the interscapular area. The heart sounds at the base were normal. P_2 was slightly louder than A_2 , but not accentuated. A systolic thrill was felt over the area where the murmur was maximum. Radial pulses were forceful, equal, and regular. Blood pressure was 134/74, subsequently 116/70 left, 120/70 right. The lungs were clear except for a small area of dullness at the left base. The breath sounds over this area were diminished and had a bronchovesicular quality. The spleen was not palpable. There was no clubbing of the fingers and no peripheral edema.

Laboratory Data.—A teleroentgenogram and cardiac fluoroscopy revealed slight cardiac enlargement to the left, presumed to be due to hypertrophy of the left ventricle. There was moderate prominence of the pulmonary conus. An angiocardigram was interpreted as indicating no enlargement of the chambers or large vessels. There was persistent visualization of the right ventricle most probably indicating an interventricular septal defect with a left to right shunt. The roentgenogram revealed a small shadow in the left lower lobe just outside the cardiac shadow thought to be due to consolidation of the lung.

The four-lead electrocardiogram was normal. There was no deviation of the electrical axis.

An ether test for right to left shunt of the circulation was negative. The maximum specific gravity of the casual specimens of urine was 1024. Tests for albumin and sugar were negative. Occasionally several red and white blood cells were seen per high-power field. Rare hyaline and granular casts were found.

The blood contained 3,500,000 red cells per cubic millimeter and 9.1 Gm. of hemoglobin per 100 c.c. There were 19,500 white cells, of which 82 per cent were polymorphonuclear leucocytes, 17 per cent small lymphocytes, and 1 per cent mononuclear cells. The erythrocyte sedimentation rate (Westergren) was 82 mm. in forty-five minutes.

The Wassermann, Kline, and Kahn tests were negative. A blood culture taken the day after admission failed to reveal any organisms.

The diagnoses were congenital heart disease, interventricular septal defect with left to right shunt, no cardiac failure, subacute bacterial endocarditis, and pneumonia or infarction of the left lower lobe.

Chemotherapy was started with sulfadiazine orally, 2 Gm. for the first dose and then 1 Gm. every four hours, six times a day. After 16 Gm. had been given, during a period of three days, the temperature, which was sustained between 102 and 104° F. during these three days, somewhat suddenly dropped to below 100° F. and then remained between 98.6 and 99.4° F. for nine days. A total of 23 Gm. of sulfadiazine had been given during the five days of this therapy; a maximum blood level of 31.0 mg. per cent was reached. On January 20 angiocardigraphy was performed, and on that day the temperature rose to 100.8° F. and daily thereafter to about 101° F. until January 26, when it rose to 104.2° F. A blood culture was then taken and revealed 58 colonies of *Streptococcus viridans* per cubic centimeter of blood.

Oral sulfadiazine therapy was reinstituted and a blood level of 15 mg. per cent was soon obtained. Fever continued and after a two-day period of from 100 to 101° F., it rose to 103° F. In conjunction with continued oral sulfadiazine, fever and foreign protein therapy with triple typhoid vaccine intravenously on successive days was started February 9. A shaking chill occurred one-half hour after each injection and lasted about one-half hour. The temperature rose to 105° F. and remained high for approximately four hours. While receiving her seventh treatment the patient developed swelling of the eyelids and a few reddish urticarial lesions on her chest. These disappeared in a few hours. Sulfadiazine was discontinued after the third injection of the typhoid vaccine because of the presence of many red blood cells in the urine. A total of 55 Gm. had been given in thirteen days. After the series of vaccine injections, the temperature dropped to from 98.6 to 100° F. for forty-eight hours. A blood culture on the first day after the last vaccine injection was sterile; a second culture

five days later revealed only 2 colonies of *Streptococcus viridans* in the blood plate but a heavy growth in the glucose broth culture. The fever returned, with the temperature fluctuating between 99 and 102° F. for twelve days. Because of the hematuria with sulfadiazine, sulfanilamide was then given, but this drug produced an increase in the fever and therefore was discontinued; 10 Gm. had been given. On March 1 the blood culture showed 7 colonies of *Streptococcus viridans* per cubic centimeter. An episode of minor pulmonary embolism occurred. Sulfadiazine therapy was reinstituted, using smaller doses (0.5 Gm. four times daily). A transfusion of 500 c.c. of whole blood was given for the anemia (hemoglobin, 66 per cent) and was accompanied by a skin reaction similar to the one after the typhoid injection. A blood culture taken March 15 revealed 32 colonies per cubic centimeter of blood.

At this point massive dose sulfadiazine therapy was considered. Dick¹³ suggested the use of 20 Gm. of sodium sulfadiazine with 30 Gm. of urea in 1,500 c.c. of distilled water daily until blood levels of from 50 to 75 mg. per cent of each were obtained. Three series of infusions at weekly intervals were advised. We decided to give this solution intravenously at a rapid rate, completing the infusion within two and two and one-half hours.

During the first infusion the patient developed the same itching and urticaria such as she had had with previous intravenous injections with other substances. They were readily controlled with $\frac{1}{2}$ c.c. of adrenalin intramuscularly. They reappeared later in the course of the infusion but responded to ephedrine, gr. $\frac{3}{8}$. After the infusion the patient became nauseated and vomited many times. The blood sulfadiazine level was 37.9 mg. per cent on the next morning. A similar intravenous infusion of sodium sulfadiazine and urea was given twenty-four hours after the first with no reaction but some nausea. The temperature curve during this time showed rises to 101 and 102° F. The following day (March 20) the blood sulfadiazine (free) was 61.9 mg. per cent and the blood urea nitrogen, 42.9 mg.; March 22 the blood sulfadiazine (free) was 33.3 mg. per cent and the urea nitrogen, 75.5 mg. per cent; and March 24 the blood sulfadiazine was 21.4 mg. per cent and urea nitrogen, 90 mg. per cent.

Because of the nausea and vomiting with consequent dehydration, on the third day following the sulfadiazine and urea treatment an intravenous infusion of saline and glucose was given and continued for four days.

The urinary output was not seriously reduced—it did drop to 540 c.c. for twenty-four hours but then increased considerably with the administration of saline and glucose infusions.

A blood culture March 23 was sterile. The temperature dropped to normal for six days following the first sulfadiazine and urea treatment and then gradually rose to 101° F. A blood culture March 29 was also sterile. The second series of infusions was started on March 29 and was identical with the first. The second infusion of this series was given the following day. The sulfadiazine blood and urine levels and the volume output of urine may be seen in Fig. 2. The maximum sulfadiazine blood concentrations reached this time were 62.1 mg. per cent free and 97.8 mg. per cent total. No untoward reaction occurred. The temperature returned to normal April 1, the day after the infusion, at which time the blood culture was sterile. A third series of infusions similar to the previous two were then given. There was a slight urticarial reaction. The blood sulfadiazine level after this third series of infusions rose to 65.4 mg. per cent free and 96 mg. per cent total.

The urine April 9 at a pH of 6.7 contained 361 mg. per cent of free sulfadiazine, 740 mg. per cent of total sulfadiazine, and 490 mg. per cent of urea N. On April 10 the urine at a pH of 6.8 contained 141 mg. per cent of free sulfadiazine, 341 mg. per cent of total sulfadiazine, and 129 mg. per cent of urea N.

The daily urinary output on one day (April 10) dropped from 2,000 to 90 c.c., but the next day rose to 1,500 c.c. On the day of the severe oliguria the urine had from 2 to 3 red blood cells per high-power field, but no sulfadiazine crystals were found.

While red blood cells and white blood cells were frequently reported in the daily urine specimens during the intensive therapy, sulfadiazine crystals were found in only two of the sixteen specimens examined and then only in small numbers.

Following the second series of infusions the temperature returned to normal and remained so from then on until the patient was discharged twenty-nine days later. During

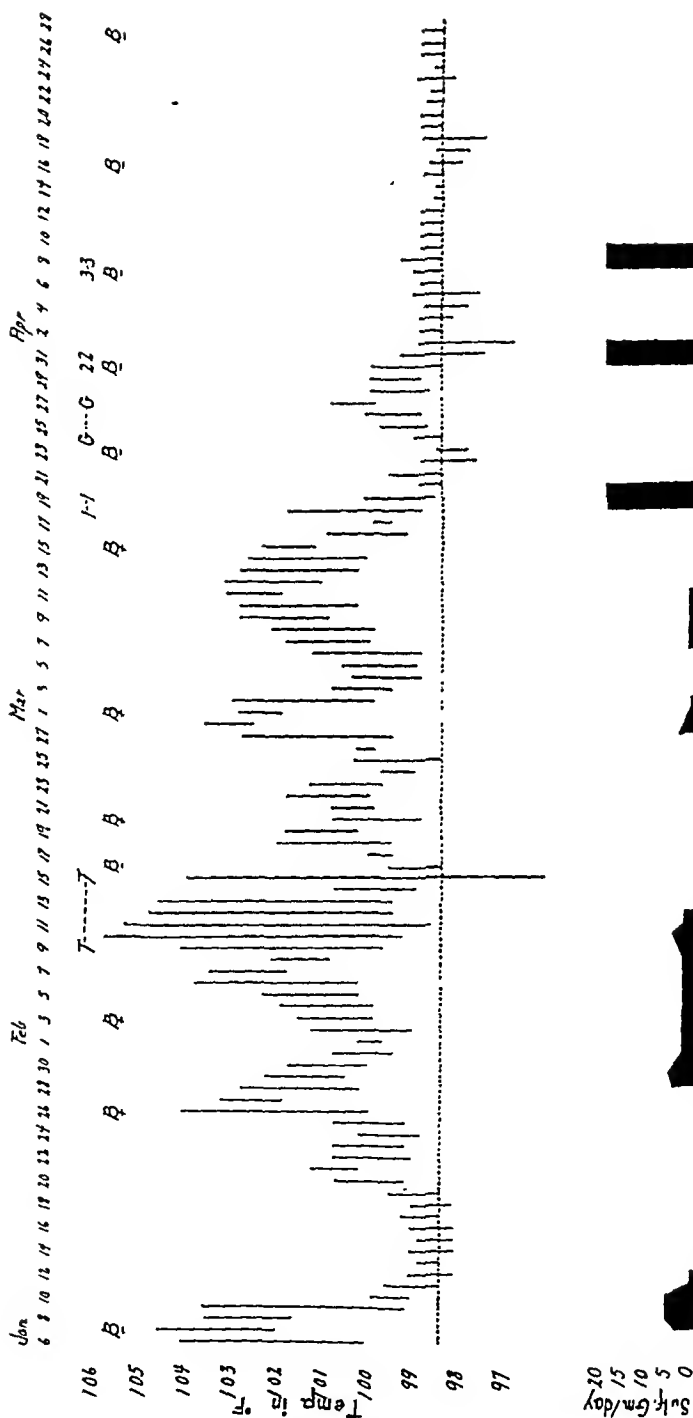


Fig. 1.—Patient's clinical course in the hospital. B, Indicates blood culture; T, daily intravenous typhoid vaccine injections; G, continuous glucose and saline intravenous infusion; I, 2-8, intravenous infusions of 20 Gm. sodium sulfadiazine and 30 Gm. urea in first, second, and third series. Hatched areas indicate daily dose of sulfadiazine.

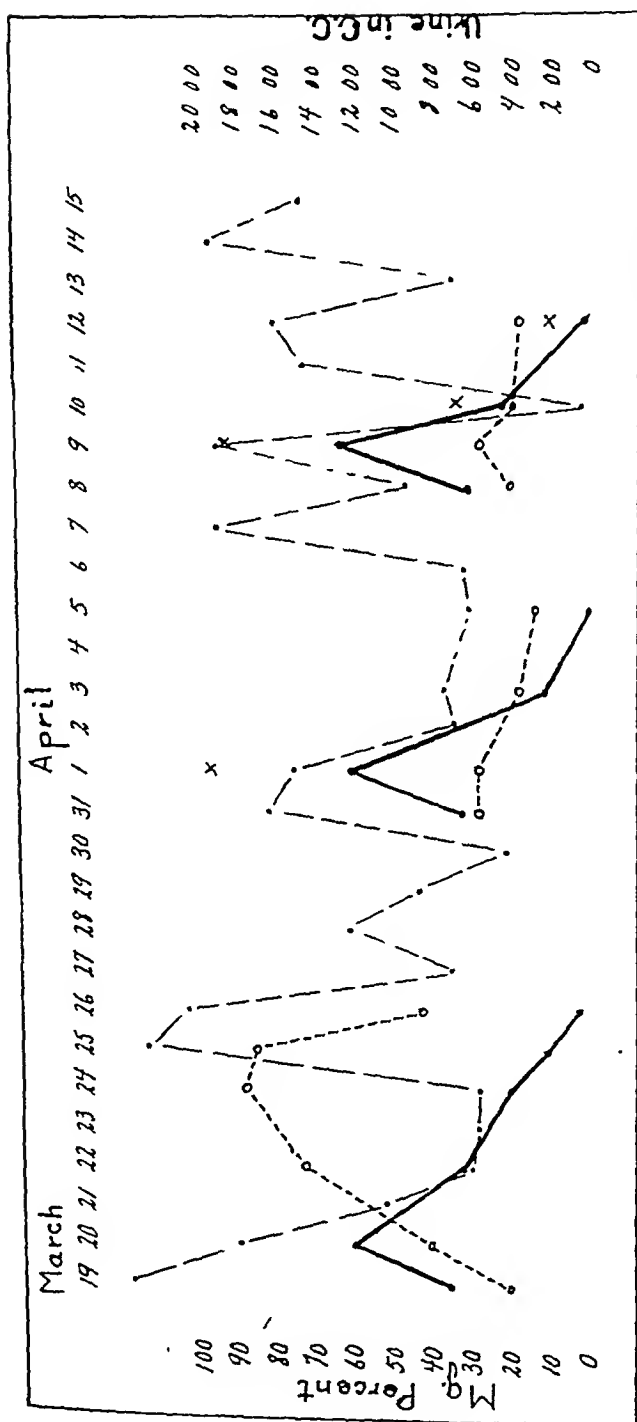


Fig. 2.—Blood sulfadiazine and urea levels and urinary volume output during the period of massive sodium sulfadiazine and urea therapy.
Continuous line, blood sulfadiazine (trco); X, blood sulfadiazine (total); small dashes, blood urea nitrogen; large dashes, daily urinary volume.

this twenty-nine day period the patient was asymptomatic except for some weakness, which gradually subsided. All blood cultures taken after the administration of the first massive dose of sulfadiazine and urea were negative.

The patient was discharged from the hospital April 29, 1943. After a few months of convalescence at home she went to work and has been working regularly since—a period of over two years. Her weight has increased to 141 pounds, a gain of 32 pounds. She has been feeling well, and at the time of the many examinations during the past two and one-half years, her temperature and pulse rate have been normal. The loud murmur is about the same. The urine has been normal; specific gravity to 1024, negative for albumin, and negative on microscopic examination. The erythrocyte sedimentation rate taken twice was normal. There has been no evidence of recurrence of the infection.

Another patient with subacute bacterial endocarditis received similar treatment with infusions of sodium sulfadiazine and urea but succumbed. Alpha and beta hemolytic streptococci in large numbers were obtained from the blood. Of particular note in this second patient too was the absence of untoward reactions of any kind with the infusions except for cardiac failure during the third series of injections. In vitro determinations revealed the organisms here to be resistant to sulfadiazine, sulfathiazole, sulfapyridine, and sulfanilamide in concentrations up to 8 mg. per cent (pH 7.3). The organisms were also found fast to sulfadiazine in concentrations of 30, 60, and 90 mg. per cent with and without urea. At 116 mg. per cent all the organisms in the culture were destroyed. This high sulfadiazine (free) concentration was found to have been reached in the patient's blood on two occasions.

DISCUSSIONS

During the course of the described treatment with massive doses of sodium sulfadiazine and urea, given intravenously, blood sulfadiazine concentrations were recorded up to 62.1 mg. per cent free and 97.8 mg. per cent total in the first patient and even higher in the second. These are from five to ten times greater than those usually reached and found efficacious in the treatment of pneumonia and other infections, as well as for bacteriostasis in vitro. Blood urea nitrogen concentrations up to 90 mg. per cent were obtained. This is equivalent to 193 mg. of urea or about .2 per cent urea. In the urine, concentrations of total sulfadiazine up to 740 mg. per cent were reached; pH, 6.7. This concentration as well as others obtained are well above the threshold for sulfadiazine crystalluria as indicated by solubility curves (Fox, Jenson, and Mudge¹⁶ and Gilligan, Garb, and Plummer¹⁷). Nevertheless, there was almost complete absence of sulfadiazine crystals in the urine. In the absence of other known causes, this increased solubility of sulfadiazine may be attributed to the use of urea and so is in accord with the findings of Sobin and co-workers.¹² It has been reported that urea may reduce the viscosity of urine.¹⁸ In this way, too, the solubility of the sulfonamide may be increased.

Little untoward reaction was encountered. Nausea and vomiting were present for a few days after the first infusion. Except for one day of oliguria, no impairment of renal function was found and none is present now, over two years later. In the second patient cardiac failure limited the further use of the infusions.

In the case reported, after other methods of treatment including the use of sulfonamide alone and also combined with typhoid vaccine injections had failed, the use of massive doses of sodium sulfadiazine and urea was promptly followed by recovery of the patient. Because of the promptness of the response and the rarity of spontaneous recovery in subacute bacterial endocarditis,¹⁹ it was felt that the sulfonamide and urea treatment was, in the main, responsible for the recovery.

The treatment has the following possible modes of action:

1. Increased antibacterial (bacteriostatic and bactericidal) power due to higher concentrations of sulfadiazine in the blood.

2. Increased penetration of vegetations in the heart by the higher concentrations of blood sulfadiazine.

3. Increased sulfonamide effect due to the urea: (a) direct potentiation of sulfadiazine²¹; (b) inhibition of antisulfonamide substances—para-aminobenzoic acid (Woods), "P" factor (Green), or other causes of sulfonamide fastness²¹; (c) increased solubility of sulfadiazine in the blood due to the presence of increased concentration of urea.

4. Increased solubility of sulfadiazine in the urine due to the urea,¹² with reduction of crystalluria and consequent renal damage following massive doses of sodium sulfadiazine.

5. Possible direct action of urea on the fibrin protectorate of the bacterial colonies in the endocardial vegetations: (a) promotion of digestion of fibrin by proteolytic enzymes (dilute solutions of urea, 2 per cent, may have such action, Ramsden)³; (b) deterrent to coagulation of proteins.³

Higher concentrations of urea than those used in the infusions were found to cause local venous thrombosis at the site of injection. The blood urea concentration can be raised by booster oral doses.

It is remarkable that no relapse occurred with such a short duration of treatment.

CONCLUSION

The use of urea as an adjunct in treatment with sodium sulfadiazine is described.

A cure of subacute bacterial endocarditis so treated, with prompt recovery, is reported.

While penicillin is preferable in cases of subacute bacterial endocarditis due to alpha hemolytic streptococcus, in infections where large doses of sulfadiazine are required the use of urea as an adjunct may be of considerable aid. This combined sulfonamide and urea therapy may be desirable in some cases resistant to treatment with penicillin.

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Scientific Program

A CLINICAL METHOD OF DETERMINING THE SPECIFIC RENAL
FUNCTIONS OF GLOMERULAR FILTRATION AND MAXIMAL
TUBULAR EXCRETION, OR REABSORPTION (AND
"EFFECTIVE BLOOD FLOW"), USING A SINGLE
INJECTION OF A SINGLE SUBSTANCE

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In a single test, accomplished with little more difficulty than is encountered in performing an intravenous glucose tolerance test plus catheterization, fairly accurate determination of glomerular filtration rate (GFR) and maximal tubular excretion or reabsorption (T_m) can be made. When diodrast (D) or p-amino hippurate (PAH) is employed for the test, determination of plasma clearance (C_D or C_{PAH}) and, therefore, of "effective renal blood flow" is also possible. The procedure for PAH is described as follows:

An amount of p-amino hippurate calculated to yield a plasma concentration of the order of 80 mg. per cent is slowly injected intravenously. Beginning ten minutes later, urine collections are made over suitable periods of time (from five to twenty minutes), and the PAH excretion per minute is determined for each period. A series of blood samples are drawn, and from the curve of declining plasma concentration values are selected corresponding to two and one-half minutes prior to the mid-point of each urinary collection period, as indicative of the average plasma level for that period. (The two and one-half minute correction is applied because of the anachronism due to excretory lag.) The excretion per minute is graphically related to the plasma concentration, using excretions per minute (UV milligrams per minute) as ordinates and plasma concentrations of unbound PAH (PF milligrams per cubic centimeter) as abscissae. A straight line is drawn to fit the points with PF values above 0.15 mg. per cubic centimeter, by inspection or by the method of least squares and is prolonged to the ordinate $PF = 0.00$.

The slope of this line is the glomerular filtration rate (GFR cubic centimeters per minute) = $\frac{\text{milligrams per minute}}{\text{milligrams per cubic centimeter}}$. The UV intercept at $PF = 0$ is the maximal tubular excretion ($T_{mPAH} = \text{milligrams per minute}$). A similar

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line applied to points with PF values below 0.05 mg. per cubic centimeter, and running through the origin, has a slope equal to the plasma PAH clearance (C_{PAH}) or "effective renal plasma flow."

While not as accurate as the more standard methods, this procedure is theoretically correct as long as there are no wide fluctuations in renal function or in blood volume during the test. The errors introduced by using a curve of decreasing plasma concentration are fewer than the technical errors of sampling and analysis. The test is more widely adaptable since it eliminates the use of inulin or other substance excreted by filtration only in order to determine filtration. Continuous intravenous infusions are not needed, and ease of analysis of PAH places the procedure within the range of any laboratory which is able to analyze for blood sulfonamide levels.

Comparisons have been made between GFR and simultaneously determined inulin clearance in fourteen cases. The average values of either method were identical. The individual comparisons presented an average difference of ± 8 c.c. per minute, or 13 per cent. Each of the values for inulin clearance represents an average of several determinations by the standard method; the average deviations for the fourteen cases were 7 per cent. The average of standard Tm values was also identical to that of simultaneously derived graphic Tm values. Individual comparisons yielded an average difference of ± 3 mg. per minute, or 10 per cent. The averaged average deviations of the individual standard Tm values was 6 per cent. Graphic values of C_{PAH} or C_D are, of course, identical to average values calculated in the usual way. The method has been applied to glucose, ascorbic acid, and hippuran figures. The accuracy of GFR determination and of the filtration fraction (the ratio $\frac{GFR}{\text{renal plasma flow}}$) is reduced where significantly large amounts of material are bound to plasma protein; and the accuracy of Tm determination is reduced in the case of substances where the Tm is small in relation to the analytic sensitivity. An ideal substance would be one whose urinary appearance is governed by glomerular filtration plus a sizable excretory or reabsorptive maximum, is not metabolized, stored in the kidney, or bound to plasma protein, and is nontoxic.

Use of this clinical method enables detection of diminution in the reserves of specific renal functions before a degree of over-all reduction in functional ability is reached which can regularly be demonstrated by present clinical tests.

A COMPARATIVE STUDY OF MERCUHYDRIN AND MERCUPURIN, ORAL AND PARENTERAL

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The diuretic and toxic properties of a new organic mercurial N-(3-methoxy-2-oxymereuripropyl) N-succinylurea, "Mercuryhydrin," were studied in 129 patients with edema. The results of this study were compared with those obtained when mercupurin was administered parenterally to fifty-two patients and orally to twenty-five.

All of the patients (206) were confined to bed, received a low-salt diet, and were weighed daily under identical circumstances. Digitalis was given to those requiring it. When weight was stabilized, one of the mercurial drugs was given; if no diuresis resulted, the drug was repeated after three days of ammonium chloride therapy. The amount of weight lost during the twenty-four hours after administration of the diuretic preparation was taken as the measure of the diuretic effect. Blood counts, urinalysis, electrocardiograms, blood nonprotein nitrogen levels, and urea clearance tests were obtained initially and at intervals during the period of observation.

Mercupurin given intravenously in 1 c.c. doses produced an average weight loss of 2.93 pounds; in 2 c.c. doses, 3.24 pounds. Mercuhydrin given intravenously in 1 c.c. doses produced an average weight loss of 2.26 pounds; in 2 c.c. doses, 3.23 pounds. Mercuhydrin was equally as effective when given intramuscularly as when given intravenously. When ammonium chloride was given as preliminary therapy, the weight loss following both mercupurin and mercuhydrin was at least twice that which occurred when the latter drugs were used alone.

Mercupurin administered orally (single dose of five tablets) was less effective than either mercupurin or mercuhydrin given parenterally. Oral mercupurin preceded by ammonium chloride produced a diuresis equivalent to that produced when mercupurin or mercuhydrin was given parenterally without ammonium chloride. Furthermore, failure to diurese occurred more often when mercupurin or mercuhydrin was administered orally.

No significant toxic effects were observed in any of the cases studied. Transient increases in the number of hyaline casts were observed following the parenteral administration of both drugs. This was the only renal change observed.

The new mercurial diuretic mercuhydrin is as effective as mercupurin and can be administered intramuscularly without pain. Oral mercupurin in the doses given is a less effective diuretic than parenteral mercupurin or mercuhydrin.

DISCUSSION

DR. ROBERT M. MOORE, Indianapolis, Ind.—I would like to report one case briefly. A 28-year-old married woman, with one child, had rheumatic heart disease when she was 8 years old. She was seen with a very severe congestive heart failure and was placed on mercupurin. She has now received 148 injections intravenously, which is apparently the one thing that is saving her life. She has an enlarged liver all of the time. I report this case only because over a long period of time she has had this medication and to date there has been no evidence of renal damage.

THE EFFECT OF THEOPHYLLINE AMINOISOBUTANOL IN THE FAILING EXPERIMENTAL HEART

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These studies were undertaken to explore, more completely, the effect of theophylline upon the heart in the course of heart failure. Observations were made on dogs.

The heart-lung preparation was used in all experiments. The right and left intra-auricular pressures were measured by direct manometry. The aortic blood pressure was optically recorded by means of a Wiggers manometer. Coronary arterial inflow was recorded using a Gregg-Green differential pressure flowmeter. Cardiac size and output were measured by the usual means. Following control observation, heart failure was induced by the careful addition of chloral hydrate (20 per cent) to the perfusing blood until myocardial dilatation and elevation of "venous pressures" were noted. Heart failure was then manifested by cardiac dilatation, fall in cardiac output (from 25 to 50 per cent), elevation of intra-auricular tension (especially that of the left auricle), and gradual decline of blood pressure. Net coronary arterial inflow in the failing heart-lung preparation was usually not altered. Theophylline aminoisobutanol (0.06 Gm.) was then added to the perfusing blood.

The administration of the theophylline to the failing heart demonstrated that:

1. The drug has an immediate effect upon the myocardium. Vigor of contraction was greatly enhanced, with concomitant diminution of intra-auricular tensions and decrease in size of the heart.
2. There was immediate diminution of pulmonary congestion.
3. No immediate increase in coronary arterial inflow occurred.

The decrease in pulmonary edema following administration of theophylline aminoisobutanol to the failing heart-lung was so striking that the phenomenon was investigated separately. Using the de Barenne modification of the heart-lung preparation, blood flow through edematous lungs could be measured, eliminating cardiac pulsatile pressure. It was found that:

1. An immediate and striking decrease in pulmonary edema and emphysema occurred after administration of the drug, with free flow of blood through the lungs.
2. Only the pulmonary edema of heart failure responded thus to theophylline aminoisobutanol. Pulmonary edema produced by the inhalation of ethyl acetate or by simple, mechanical chronic passive congestion (without heart failure) did not respond to theophylline.

DISCUSSION

QUESTION.—Has there been any success in the treatment of emphysema without heart failure?

QUESTION.—I would like to ask if any method other than chloral hydrate produced heart failure.

DR. EMMET B. BAY, Chicago, Ill.—I would like to ask what type of reservoir was used in the last experiment. At what pressure was the blood flowing into the pulmonary artery?

DR. SMITH (closing).—There may be some application of this method to the treatment of emphysema without heart failure. It is well known that the effect of this drug upon the lungs, particularly in cardiac asthma, is quite striking. It has been noted to be effective in

bronchial asthma. I would imagine that, if emphysema were complicated by chronic bronchitis, this drug would have some beneficial effect.

Chloral hydrate was the only drug used to produce heart failure.

In answer to Doctor Bay, the type of reservoir used in the last experiment was at fixed elevation (33 cm. above the right auricle). In ordinary heart-lung preparation, this elevation of the venous reservoir assures one of an adequate blood flow into the heart.

EFFECT OF STIMULI ORIGINATING IN THE UPPER ABDOMEN IN THE CAUSATION OF REFLEX SHORTENING OF THE ESOPHAGUS

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There are clinical reasons for assuming that some cases of hiatus hernia may be caused by reflex shortening of the esophagus consequent upon impulses arising from the upper abdomen. In a previous paper, it was demonstrated that electrical stimulation of the vagus nerve caused sufficient shortening of the esophagus to pull the stomach up to the hiatal orifice. In these experiments the chest was closed, and we were not able to pull the cardiac end of the stomach through the hiatal opening. The present series of experiments were performed on the anesthetized dog with the left chest opened and a lever attached to the esophagus. Movements of the esophagus were recorded upon a revolving drum. Manipulation of the liver, pressure upon the gall bladder, and distention of the gall bladder caused a reflex shortening of the esophagus similar to that obtained by electrical stimulation of the vagus. Reflexes were obtained elsewhere in the gastrointestinal tract. A hiatus hernia occurred with slight pressure upon the abdomen before it was opened, and during the experiment with the abdomen opened it was reproduced again by a shortening of the esophagus due to reflex stimulation or electrical stimulation.

GASTROCOLIC FISTULA—AN EXPERIMENTAL STUDY

R. JOHN F. RENSHAW, M.D. (BY INVITATION), R. M. KISKADDON, M.D.
(BY INVITATION), AND FREDERIC E. TEMPLETON, M.D., CLEVELAND, OHIO

Patients having gastrocolic or gastroenterocolic fistula present a combination of symptoms consisting of nausea, vomiting (often with fecal material), diarrhea, nutritional deficiency, and cachexia. In some patients the stools are clay colored and contain undigested food, neutral fats, and fatty acid crystals. The patients may also develop anemia, sometimes of the hyperchromic macrocytic type. The usual explanation of the syndrome is that food passes directly from the stomach into the large intestine and short-circuits the small bowel. A few workers disagree with this explanation. For instance, Pfeiffer and Kent, on the basis of fluoroscopic observation, record that the presence of undigested food "results from exceedingly rapid transport of food through the stomach and intestine and is probably due to irritation of the tract from the presence of colonic contents from the stomach and bowel." Atwater, Butt, and Priestly

admit that reflux of fecal content plays a part in causing the syndrome, but they state that the mechanical factor (shunting of food out of the stomach into the colon) is foremost.

In view of these conflicting opinions, we observed the flow of barium mixtures in nine patients and in six dogs having gastrocolic, duodenocolic, and gastroenterocolic fistulas. In four patients, as well as in the experimental animals, the major portion of the aliment passed down the small intestine instead of entering the colon through the fistula. The human small intestine appeared abnormal, as in the so-called "deficiency state." Roentgenologic findings followed the descriptions of most roentgenologists: The gastrocolic fistula may be overlooked by giving a barium mixture orally but is usually recognized during administration of a barium enema. In one dog, at roentgenologic examination, we not only watched the barium leave the stomach through the pylorus and flow through the small intestine and colon, but also observed a portion of the mixture return to the stomach through the fistula and emerge again into the small intestine through the pylorus.

The studies confirm the opinion of Pfeiffer and Kent that in gastrocolic, gastroenterocolic, and duodenocolic fistulas, much of the barium mixture follows the small intestine and does not flow directly into the colon. The deficiency pattern of the small intestine seen by roentgenologic examination, along with the presence of neutral fats and fatty acid crystals which were observed in the stools of three patients and all the dogs, indicates that the small intestine is injured. It is suggested that the fecal current, flowing into the stomach and upper small intestine through the fistula, injures the mucosa so that the intestine cannot perform the normal processes of absorption and secretion. Experiments now in progress are designed to study further the latter possibility.

DISCUSSION

DR. E. L. DEGOWIN, Iowa City, Iowa.—In patients with gastrocolic fistula, when the fistula is repaired, the patient is improved and the condition seems reversible. I wonder if any observations were made and whether any histologic evidence is present to show that the damage produced in the experiments is reversible.

QUESTION.—How long did the dogs survive following surgery?

DR. GEORGE E. WAKERLIN, Chicago, Ill.—Since the dogs used in this study showed a macrocytic hyperchromic anemia, the possibility that this type of animal might serve as a means of assaying preparations containing the antipernicious anemia principle comes to mind. Have you studied the effect of the antipernicious anemia principle in these dogs?

DR. KISKADDON (closing).—In regard to the first question, "Is the pathologic process reversible?" we feel that there is definite evidence, as presented in the slides of the pathologic specimens, that these are chronic ulcers. We do not have evidence, as yet to indicate whether the dogs can be brought back to normal, because we have been primarily interested in the direction of barium flow and the changes which take place in the intestine. We are investigating these dogs gastroscopically and are studying other dogs into which we have introduced permanent cannulas. We thereby hope to be able to observe the formation and retrogression of the ulcerative process.

In answer to the second question concerning the length of life of these dogs with internal fistulous communications, we must explain that it varies with the type of fistulous connection. Dogs with gastrocolic fistulas survive about four months. However, dogs with

gastrojejuno-colic fistulas usually survive about two months. The dog with a duodenocolic fistula died one month after the fistula was established. This brings up the point as to what the protective factor may be in dogs with gastrocolic fistulas in contrast to dogs with gastroenteric fistulas. It is possible that in the dogs with gastrocolic fistulas the colonic micro-organisms are killed by the acid of the stomach, but we do not know that this is true. In contrast, the acid component would not be present in dogs with gastroduodenal fistulas, and therefore there should be no means of inhibiting the destructive action of the bacterial flora on the upper intestinal mucosa.

As you can see, our experiment is still in its initial phase, and we are unable to answer the last question concerning the availability of such dogs in the bio-assay of the antipernicious anemia factor.

SULFATHALIDINE—FURTHER STUDIES ON CLINICAL, CHEMICAL, AND BACTERIOLOGIC EVALUATION IN INFECTIOUS DISEASES OF THE COLON*

MICHAEL H. STREICHER, M.D., CATHERINE GRENCI (BY INVITATION), AND
NANCY CLAAR (BY INVITATION), CHICAGO, ILL.

Experimental evidence obtained shows that sulfathalidine has bacteriostatic properties, that it is nontoxic in man, and that it is superior to any sulfonamide used in the colon. It is effective in doses of 1 Gm. orally three times daily. This dose may be maintained for several months without producing deleterious effects. Prolonged administration and increased doses of this agent do not elevate the blood concentration above from 1 to 1.5 mg. per 100 c.c. of blood.

The chemical determinations of sulfathalidine in the stool (total amounts) tend to show that, when oral intake is increased to 12 Gm. daily, the recovery of the drug in the stool is not increased correspondingly.

Inasmuch as the blood and the urine concentration levels remain unchanged, this experiment tends to show that large doses of sulfathalidine are not utilized in the colon.

Another experiment designed to study the total recovery of the drug in the stool after the intake of the last dose shows that 90 per cent of sulfathalidine is accounted for on the seventh day.

Bacterial counts demonstrated a decrease in total number and in the number of the coliform group, the staphylococci, and the streptococci.

Clinically the patients who are ill with chronic ulcerative colitis, bacillary dysentery, or *Giardia lamblia* may be treated with sulfathalidine to advantage. In amebic dysentery the drug is used to curtail the activity of secondary invaders.

Patients with diarrhea register definite improvement, the stools become more formed and odorless, the number of evacuations are decreased, the blood in the stool disappears, the cramping in the abdomen is alleviated, the tenesmus in the rectum subsides, and in general the patient eats better and feels better.

*The work on this research problem was made possible through a grant sponsored by Sharp & Dohme, Inc., Glenolden, Pa.

DISCUSSION

DR. RALPH C. BROWN, Chicago, Ill.—I think it would be a mistake for the impression to become widespread in the profession that the sulfonamides are of great value in the treatment of nonspecific ulcerative colitis. There is no question about the ability of these drugs to lower the bacterial counts decisively. This was first proved very thoroughly in the case of sulfaguanidine, very careful bacterial counts having been made on a series of cases. For many years at the Presbyterian Hospital we have made an intensive study of a large series of patients with ulcerative colitis. With each successive sulfonamide as it has appeared, we have hoped that finally a therapeutic agent had been devised that would demonstratively affect the course of the disease. However, in our experience we have not been able to convince ourselves that any of the sulfa preparations actually do so. I think that anyone who has had very much experience with nonspecific ulcerative colitis is likely to feel that success in the treatment of these cases depends very largely upon the stage at which they are seen and effectively treated, and when I say effectively treated I do not mean that we have anything even approaching a specific type of treatment. These patients must be treated in much the same manner as those with pulmonary tuberculosis in the sense that they require a high caloric diet and particularly a long period of bed rest, with large amounts of vitamins B and C. If they are seen early in the course of the disease, it will be found that surprisingly good results can be obtained in most cases if the duration of treatment is determined not alone by the clinical condition of the patient, but chiefly by the presence or absence of inflammatory changes in the bowel wall as shown by the proctoscope and by x-ray examination.

DR. STREICHER (closing).—I appreciate the comments of Doctor Brown. I am sure that sulfathalidine alone is not the answer to the problem. We have been working on this problem for many years and have had a good many experiences. In the extremely acute cases we have used blood transfusions, liver extract, and all other supportive measures.

As far as judging the progress of the disease by clinical observation alone, we do not depend on that entirely; we re-proctoscope our patients. Some few years back we published a report in the *Journal of Digestive Diseases* on 200 patients proctoscoped yearly over an eight-year period. I quite agree with Doctor Brown that sulfonamides alone are not the entire answer, but sulfathalidine is the best we have of the sulfa group at this time.

THE PRESIDENT'S ADDRESS BY WILLIS FOWLER was presented at this point in the program.

THE DETECTION OF DANGEROUS CARRIERS OF HEMOLYTIC STREPTOCOCCI BY THE MEASUREMENT OF THEIR "STREPTOCOCCAL OUTPUT"

MORTON HAMBURGER, JR., M.D.,* CINCINNATI, OHIO

Students of streptococcal infection have recognized, for several years, that all throat carriers of beta hemolytic streptococci are not equally capable of transmitting infection to others. However, no means of differentiating "dangerous" from "innocuous" carriers have been available.

A solution to this problem was sought by measuring the number of hemolytic streptococci expelled into the environment by soldiers hospitalized at the Station Hospital, Camp Carson, Colorado, with streptococcal tonsillitis, pharyngitis, and scarlet fever. Contamination of the bed clothing was employed as an index

*These investigations were carried out by the Commission on Air-Borne Infections, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, United States Army.

of the "streptococcal output" and was measured quantitatively by pinning a small cloth patch to the bottom sheet overnight. The patch was then removed and immersed and shaken in broth, an aliquot of which was used for making a blood agar pour plate. The number of colonies of hemolytic streptococci which developed after incubation represented the "streptococcal output."

This test, performed many times in more than 400 patients, showed that the number of hemolytic streptococci expelled bore no relation to the number of these bacteria on the throat culture plate. However, patients with strongly positive nose cultures dispersed large numbers of the organisms, whereas those with negative nose cultures expelled very few. Hospital cross-infections, infections in barracks, and a food-borne epidemic of streptococcal pharyngitis and tonsillitis were traced to carriers with positive nose cultures who exhibited high "streptococcal outputs."

It is concluded that carriers of beta hemolytic streptococci whose nose cultures are positive represent a more dangerous group than those in whom the streptococci remain confined to the throat.

DISCUSSION

MAJOR F. S. COOMBS, JR., Chanutte Field, Rantoul, Ill.—I think that the paper is very stimulating. We, too, have been interested in the same problem. I wonder if there is any difference in the length of the disease when patients are nose carriers rather than throat carriers.

DR. M. A. BLANKENHORN, Cincinnati, Ohio.—I would like to ask whether Doctor Hamburger found carriers in other persons in the hospital. He has studied bed patient carriers, but what about persons who worked about the ward and ministered to these patients?

I would like to ask if he has an opinion regarding the value of the gauze face mask in preventing dissemination.

DR. IRVINE H. PAGE, Cleveland, Ohio.—I would like to ask Doctor Hamburger whether he knows anything about the use of D.D.T. or aerosols of propylene glycol in preventing the spread of hemolytic streptococci. Is there anything known about which Lancefield grouping the streptococci belong to?

DR. HAROLD LUETH, Evanston, Ill.—Was any attempt made to study the susceptibility of persons affected to see whether or not they were uniformly susceptible or whether there was any variation among the group?

DR. HAMBURGER (closing).—In reference to Major Coombs's question as to whether a streptococcal infection has a longer clinical course in a patient whose nose, as well as throat, is positive, we have no specific data on this point. However, in general, hospitalized patients with positive nose cultures tend to be sicker than those in whom the nose is not invaded. At Camp Carson about 65 per cent of the men hospitalized with hemolytic streptococcal infection had positive nose cultures, and some of them expelled great numbers of streptococci during the early stage of their disease.

As to the applicability of these facts to ambulatory, as well as to hospitalized, carriers, they apply to both. Actually, most dangerous carriers are infected individuals. Captain H. M. Lemon and I saw a good many soldiers this spring at Fort Lewis, Wash., who may or may not have had fever but who had mild streptococcal infections and were picked up as nasal carriers on surveys. They had made no complaint, but physical examination and antibody studies showed them to be people who were infected but who were going about as any normal person. The average dangerous nasal carrier disperses large numbers of streptococci for a very short time. On the other hand, there are a few, probably not more than 1 per cent, who continue to expel large numbers of streptococci for weeks or months. We have seen a few

such carriers. Apparently, when epidemics occur, nasal carriers increase in number, and though most are dangerous for a short period only, there are more and more of them.

To answer Doctor Blankenhorn, I think I have covered the point about bed patients. The most detailed studies were necessarily done on hospitalized patients, but we have also been able to follow the carrier state by studying carriers in barracks. The carrier of streptococcus type 46 was picked up on a survey and was entirely asymptomatic.

About masks, the problem of how these streptococci get around is a study all its own. For reasons I have not time to discuss, I do not believe that adequate masking is the answer to the problem. However, there are masks which will effectively prevent the spread of microorganisms expelled during talking, coughing, and, to some extent, during sneezing.

As to propylene glycol, we have been interested in this compound which was originally developed by Dr. O. H. Robertson, the Director of the Commission on Air-Borne Infections. For certain technical reasons, propylene glycol is not the answer. We have studied triethylene glycol in reference to its effect upon hemolytic streptococci in hospital wards, and we have found that it produces a substantial reduction in number of streptococci. To what extent, reducing the number of organisms in the air will reduce the incidence of infection requires further study.

The final question regarding the susceptibility to streptococcal infection of the patients who developed cross-infections on the wards is an interesting one. The only real answer we have is statistical. We were dealing with young soldiers all of the same age group, living under the same conditions, and receiving essentially the same training. It is true that under certain circumstances certain types of patients are more susceptible to streptococcal infection; measles convalescents, for example. Some of our cross-infections did take place on measles and German measles wards.

ANTIPNEUMOCOCCUS HYALURONIDASE ACTIVITY IN HUMAN SERUM; RISE IN TITER FOLLOWING PNEUMOCOCCUS BACTEREMIA WITH PURULENT INFECTION OF PLEURA AND SYNOVIA

PRELIMINARY REPORT

ROBERT T. THOMPSON, M.D. (BY INVITATION), AND
M. A. BLANKENHORN, M.D., CINCINNATI, OHIO

The identity of hyaluronidase and spreading factor, in that all hyaluronidases possess the power of spreading, has been described. Since spreading factors have been shown to be important in infection, the *in vitro* effect of human serum in health and during pneumococcal infection upon the activity of three pneumococcus hyaluronidases was investigated. A modification of the mucoprotein clot prevention test described by McClean in 1943 was used to study the antipneumococcus hyaluronidase activity of human serum.

The serum of fifty healthy human beings was compared with the serum of a patient, A. T., who was convalescing from type II pneumococcus endocarditis. The antitype II pneumococcus hyaluronidase activity of the serum of two healthy persons was equal that of A. T., four were one-half that of A. T., ten were one-fourth that of A. T., sixteen were one-eighth that of A. T., seven were one-sixteenth that of A. T., nine were one-thirty-second that of A. T., and two were one-sixty-fourth of A. T.

From the Department of Medicine and the Department of Biochemistry, College of Medicine, University of Cincinnati.

Serial sera of five patients with pneumonia each showed critical rises in titer of antienzyme activity against three types of pneumococcus hyaluronidase which were synchronous and commensurate and not related to the type of pneumococcus causing the pneumonia. Three enzymes (types I, II and VII) were tested for each patient, so there was a total of fifteen separate rises in titer. Thirteen of these rises in titer were eightfold or greater; two were fourfold. Each of these patients had pneumococcus bacteremia, three with empyema and one with arthritis.

This rise of antipneumococcus hyaluronidase titer could not be provoked by administration of therapeutic antipneumococcic serum in two patients. Negligible amounts of antitype II pneumococcus hyaluronidase activity were found in therapeutic type II antipneumococcic serum.

Bacillus welchii antitoxin which had high anti-*B. welchii* hyaluronidase activity was found to have negligible effect against type I and type II pneumococcus hyaluronidase.

It is concluded that human serum possesses antipneumococcus hyaluronidase activity and that this activity is not type specific. Five patients showed critical rises of antipneumococcus hyaluronidase activity subsequent to pneumococcus bacteremia; three of these had purulent infection of pleura and one of synovia. Therapeutic type II antipneumococcic serum possesses negligible amounts of antipneumococcus hyaluronidase activity. This finding probably applies to other types of therapeutic antipneumococcic serum since the antipneumococcus hyaluronidase activity of human serum was found not to be type specific.

THE EFFECT OF SUPERIMPOSED BACTERIAL PNEUMONIA ON THE SEVERITY OF SUBLETHAL INFECTION WITH INFLUENZA VIRUS*

CARL G. HARFORD, M.D., MARY RUTH SMITH, M.S. (BY INVITATION), AND
W. BARRY WOOD, JR., M.D., ST. LOUIS, MO.

It is thought that most of the deaths in the pandemic of influenza of 1918-1919 were due to bacterial pneumonia superimposed on a primary viral infection of the lung and that chemotherapy will be effective in controlling the pneumonia if another pandemic occurs. However, Taylor has shown that reinstitution of fluids into the respiratory passages of mice suffering from a sublethal infection with influenza virus converts the infection into one from which the animals die. Because of this observation he has suggested that the bacterial pneumonia may greatly increase the severity of the viral infection. If such synergistic action occurs, it may be anticipated that treatment in human epidemics will be difficult, since the viral infection is not susceptible to any known chemotherapeutic agent.

In this study, infection with influenza virus was produced in mice by intrabronchial inoculation with standardized dilutions of virus. Several days later, when viral lesions had developed, the mice were allowed to inhale fine droplets

*This investigation was aided in part by the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

of heavy suspensions of virulent type I pneumococci or hemolytic streptococci. The bacterial exposure caused fatal pneumonia and bacteremia in about five days. Normal control animals inhaling the same bacteria survived without clinical disease, but their lungs were shown to have many organisms which disappeared within a few hours. One-half the animals with combined infection were given sulfonamide therapy beginning twenty-four hours after inhalation of the bacteria, that is, after the superimposed bacterial pneumonia was well established. The treated animals survived and showed sublethal viral lesions when examined two weeks later.

In these experiments, superimposed bacterial pneumonia did not convert a sublethal viral infection into a lethal one. Under the conditions studied, the bacterial infection did not have the same effect as the reinstallation of fluid described by Taylor. Furthermore, the bacterial pneumonia in the presence of viral lesions was controlled by sulfonamide chemotherapy. These observations suggest that chemotherapy will be of value in treating secondary bacterial pneumonia complicating epidemic and pandemic influenza.

DISCUSSION

DR. H. NECHLES, Chicago, Ill.—We can confirm these results by our experience on dogs with distemper which we have treated for the last ten years with sulfonamide drugs. Distemper in dogs is a virus disease. In the beginning it is a pure virus, but, as soon as the nasal and conjunctival flow begin, we have a mixed infection with pneumococci and cocci of all sorts. These dogs are benefited by sulfonamide, glucose, and saline given subcutaneously. We have been quite successful with this treatment. We believe that, as in the case of the human patient, it is the suppression of the secondary infection which saves the lives of these dogs.

THE SIGNIFICANCE OF THE ACCESSORY SPLEEN

GEORGE M. CURTIS, M.D., AND DAVID MOVITZ, M.D. (BY INVITATION)
COLUMBUS, OHIO

When *accessory spleens* are present in patients with congenital hemolytic icterus or primary thrombocytopenic purpura, the end result of splenectomy is influenced by whether or not they are found and removed at the original operation. This was surmised in 1928 by Morrison, Lederer, and Fradkin for primary thrombocytopenic purpura. Subsequent findings of accessory spleens associated with recurrence further support their original suggestion.

The reported frequency of accessory spleens at necropsy ranges from 10 to 35 per cent and at operations on patients with splenic disease, from 20 to 44 per cent. A greater frequency among the young has been noted.

Of 178 consecutive patients in our series, operated for various splenic disease, fifty-six, or 31.4 per cent, presented accessory spleens. The greatest incidence was found in patients of the first decade with congenital hemolytic icterus, 57.1 per cent. Forty-four per cent were found in patients of the first decade with primary thrombocytopenic purpura. The incidence decreased with age. The number of accessory spleens per patient varied from one to ten, twenty-six patients presenting only one accessory. Single as well as multiple accessories occurred predominantly in the young.

The 131 accessories occurring in fifty-six patients were distributed in the following percentages: hilus, 54.2; pedicle, 25.1; omentum, 12.2; retroperitoneum, 6.1; splenocolic ligament, 1.5; and bowel mesentery, 0.75. Accordingly, these regions are routinely searched subsequent to the actual splenectomy. In the female the left adnexa should be palpated.

Numerous accessories, up to 400, found scattered throughout the peritoneum, including parietal and ventral mesogastric layers, apparently arise by autotransplantation of splenic tissue scattered about by hemorrhage from a ruptured spleen. Buchbinder and Lipkoff (1939) designated this phenomenon as *splerosis*, distinguishing it from the usual accessory spleens because of the mechanism of formation and the characteristic microscopic structure. As a consequence, splenectomy should be accomplished without spilling splenic tissue, as might occur by tearing the spleen near its adhesions or severing its pedicle too close to the hilus.

Accessories occurred in but a single location in 85.7 per cent, and never in more than two locations. The hilus always constituted one location in each instance of double incidence.

The occurrence as well as the various locations of accessory spleens are clarified by study of the splenic embryologic mechanisms: failure of splenic anlagen to fuse, splenic notches and lobules representing partial fusion, co-existing accessories supplied by the splenic artery, the dorsal mesogastrium subjacent to the splenic area developmentally carrying accessories to distant locations, and the attachment of splenic anlagen to the contiguous genital ridge resulting in serotal or pelvic accessories.

Two presented cases of recurrence after splenectomy and two more from the literature indicate that unremoved accessory spleens caused the recurrences. Three cases were of recurrent thrombocytopenic purpura, with one accessory observed but not removed and two with pathologically active accessories found at necropsy. One was a recurrent congenital hemolytic icterus relieved by surgical removal of accessory spleens.

A pedunculated accessory spleen may cause acute abdominal symptoms by infarction resulting from torsion of its pedicle. Surgical removal is then indicated.

DISCUSSION

DR. ARMAND QUICK, Milwaukee, Wis.—I should like to ask how often idiopathic thrombocytopenic purpura is seen in children.

DR. OVID O. MEYER, Madison, Wis.—I would like to ask whether or not these accessory spleens are of any importance, other than in the matter of torsion which was mentioned, unless there is hypertrophy? It is our impression that these accessory spleens are often very small and easily overlooked and are not important until months or years later when they hypertrophy. They then have an undesirable function.

DR. MOVITZ (closing).—The frequency of recognition of idiopathic thrombocytopenic purpura partly depends on the practices of certain medical centers. We see it relatively frequently. The primary thrombocytopenic purpuras are among the most common indications for splenectomy.

Regarding the small size of most accessory spleens, it appears to be true that recurrence due to an accessory spleen does not occur except after an interval of time. That is the usual history in those few recurrences in which an accessory spleen was demonstrated. The cases reported have all demonstrated considerable pathologic changes in the accessory. As to the size of the accessory spleen causing the recurrence, some were as small as 2 or 3 cm., and one weighed but 12 Gm. It would appear that the minuteness of size of an accessory spleen does not eliminate it as a cause of recurrence at a future time.

OBSERVATIONS ON THE EFFECT OF MASSIVE DOSES OF IRON GIVEN INTRAVENOUSLY TO PATIENTS WITH HYPOCHROMIC ANEMIA

ANNE TOMPKINS GOETSCH, M.D. (By INVITATION), CARL V. MOORE, M.D., AND
VIRGINIA MINNICH, M.S. (By INVITATION), St. Louis, Mo.

Massive doses of iron (from 0.608 to 1.32 Gm. iron as colloidal ferric hydroxide or colloidal ferric oxide) have been given intravenously in one infusion to eight different patients with hypochromic microcytic anemia. One patient was given a second injection after an interval of four months, so that the administration was made nine times.

The following observations were made:

1. Reticulocyte responses in each instance exceeded those expected with oral therapy. Three additional patients who received smaller doses showed reticulocyte rises higher than the average responses reported by Heath after optimal oral administration. This suggests that "optimal" oral therapy does not provide a maximal stimulus to the outpouring of reticulocytes from the bone marrow. Three control subjects failed to develop a reticulocytosis when given comparable amounts of iron intravenously.

2. The average rate of hemoglobin regeneration per 100 e.e. of blood per day was 0.224 Gm.; the lowest, 0.16 Gm.; and the highest, 0.27 Gm. These figures indicate the rise which occurred from the day of iron administration to the time at which the rate of hemoglobin increase was obviously becoming slower. Since correction was not made for blood lost by three of the patients during the recovery period, figures for the rate of hemoglobin formation are lower than they otherwise would have been. They are distinctly greater than those usually observed following oral therapy but no greater than is found in an occasional patient. The data suggest that the fastest rate of hemoglobin regeneration which can be stimulated by iron in patients with hypochromic anemia approximates 0.3 Gm. per 100 e.e. per day.

3. Calculations indicated that from 71.8 to 99.7 per cent of the injected iron was used for the synthesis of hemoglobin. These figures are lower than they would have been if several of the patients had not lost blood during the recovery period. The observation of other workers that parenterally administered iron is almost completely retained by the body and converted into hemoglobin was confirmed.

4. Toxic reactions to the injected iron were mild or absent in two patients. In most instances the patients developed nasal stuffiness, paresthesias, palpitation, nausea, transient fall in blood pressure, and, later, manifestations of fever and weakness. In three instances these symptoms progressed to an alarming degree, and therapy was interrupted. In all patients the toxic symptoms subsided within a few hours. The toxic manifestations to massive intravenous iron administration are great enough to contraindicate its therapeutic use.

DISCUSSION

DR. IRVINE PAGE, Cleveland, Ohio.—What would be the effect if you used an organic iron preparation, ferric gluconate, and not a colloid? Would part of the effect be due to the introduction of the colloid particles as well as to the iron in solution?

QUESTION.—What is the pH of the injected fluid?

QUESTION.—I should like to ask whether there were variations in the rate of administration and what the rate was.

QUESTION.—I would like to ask if you have seen patients who were so intolerant of oral administration that they would consider the parenteral use of iron in smaller doses.

DR. H. NECHELES, Chicago, Ill.—Have you information about a French parenteral iron preparation? It was a water-clear solution and, as I remember, the dose was quite small. Colloidal iron is quite a strong protein precipitant. Is the reticulo-endothelial system affected?

DR. GOETSCH (closing).—"Clear iron," or metallic iron, in simple solution has been used in small doses for parenteral injection, usually by the intramuscular route, although it has also been given intravenously. Toxic effects observed following only from 5 to 10 mg. doses intramuscularly were similar to those we have observed using colloidal iron; this is one basis for our impression that these toxic effects are so-called "heavy metal effect" rather than a result of the colloidal suspension per se. When simple iron solutions are given to dogs in doses of 1 mg. or more per kilogram of body weight, twelve hours or more are required for serum iron values to return to normal, whereas the return to normal is prompt after colloidal iron has been given. This more rapid disappearance of colloidal iron from the blood may explain the fact that massive doses could be given to our patients in a single intravenous injection.

Animal experimentation has shown that the reticulo-endothelial system is the site of storage of injected iron. We did no studies to demonstrate blocking of the reticulo-endothelial system in our patients. There were no demonstrable emboli. One patient complained of severe chest pain during the administration of iron, but no signs of pulmonary embolism appeared.

The solutions contained approximately 3.5 mg. per cubic centimeter; their pH varied from 3.5 to 3.8; the rate of administration was from 10 to 25 drops per minute; and from three to five hours were required for administration of total dose.

Reticulocyte responses have been reported after very small doses of iron, 10 mg. in several instances. We found this to be true in our three patients who had toxic reactions necessitating discontinuation of the injection. In these cases it was possible to obtain a second reticulocyte peak with subsequent oral therapy. Significant rise in hemoglobin was not observed after small doses, since any apparent rise was within the usual fluctuations in hemoglobin levels. Maximum reticulocytosis was observed only following the larger doses of iron.

The toxic effects of small doses of colloidal iron, either intramuscularly or intravenously, are not great enough to contraindicate its use but are unpleasant enough to suggest that its use be reserved for the rare patient who cannot tolerate oral iron therapy. Furthermore, it was our experience that small intravenous injections caused thrombosis of the vein as readily as did larger doses.

THE ACTION OF VARIOUS DRUGS ON THE PROTHROMBIN
OF THE BLOOD

ARMAND J. QUICK, M.D., MILWAUKEE, WIS.

In order to test whether large doses of synthetic vitamin K will increase the prothrombin of the blood, menadione and two water-soluble derivatives, Hykinone* and Synkayvite† were administered to rabbits, dogs, and human beings, both orally and intravenously. No change in the prothrombin was observed after giving one large single dose.

In a subject who has a consistent prothrombin a little less than 50 per cent of normal (from fifteen and one-half to sixteen seconds) but is otherwise entirely normal and never has had abnormal bleeding, 40 mg. of hykinone intravenously had no effect on the prothrombin level. The mother and a sister likewise have a prothrombin of 45 per cent. This indicates that the condition of a fixed lowered level of prothrombin can be hereditary and congenital. The prothrombin concentration of the blood, whether normal, congenitally low, or reduced by permanent liver damage, is fixed and apparently cannot be altered even by large doses of synthetic vitamin K.

Link and his associates have reported that caffeine, theobromine, and theophylline bring about a hyperprothrombinemia and also counteract the hyperprothrombinemic action of dicumarol. In the present study it was found that single doses of 200 mg. of the three methylxanthines neither elevated the prothrombin nor inhibited the action of dicumarol in rabbits and dogs.

Recently, Pirk and Engelberg published results showing that daily doses of 0.33 Gm. of quinine sulfate given to normal individuals caused a drop in the prothrombin time of from five to twelve seconds. These authors used Russell viper venom as the thromboplastic reagent for determining prothrombin. In the present work in which my method for prothrombin was used, no reduction in prothrombin of the blood was found after administering 0.33 Gm. of quinine sulfate daily for eight days.

The discrepancies in results are probably due mainly to differences in the procedures employed for determining prothrombin. It is questionable whether true hyperprothrombinemia is ever found or can be induced. All present evidence so far advanced for it is based on the prothrombin time of highly diluted plasma, which is necessarily artificial and empirical since the physicochemical equilibrium is profoundly disturbed.

DISCUSSION

DR. FREDERICK DEY, Chicago, Ill.—We were quite concerned about the recent report that methylated xanthines are capable of increasing the coagulability of the blood in the experimental animal. We use these drugs quite extensively in the treatment of cardiac diseases, and while we have had no reason for feeling that they promoted thrombosis in our patients, we thought it only wholesome to study the effect of these drugs upon the clotting mechanism of the human being.

*Abbott Laboratories, North Chicago, Ill.

†Hoffmann-La Roche, Inc., Nutley, N. J.

A group of normal patients who had never before received methylated xanthines were admitted to the hospital. During the initial week of the study, daily clotting times, heparin curves, and prothrombin times, using both whole and diluted plasma, were run on the patients. After the normal was established, methylated xanthines were administered by various routes for a period of one week. Again, daily studies of the clotting times, heparin curves, and prothrombin times were made. We found no variations from the normal after the administration of these drugs.

Similar studies of clotting activity were made on a group of fifteen patients who had been taking methylated xanthines for a period of six weeks up to twelve years. Again, there was no variation from the normal.

Finally, we repeated the work on dogs, similar to the study of Doctor Quick's, and found no change in clotting activity.

DR. GEORGE E. WAKERLIN, Chicago, Ill.—In a study made for another purpose, our research group administered vitamin K in a daily oral dose of 60 mg. for six months to six dogs with renal hypertension. Limited studies of the prothrombin levels of these animals showed no significant change from the normal.

DR. E. V. ALLEN, Rochester, Minn.—This question is outside the proposition, but I would like to ask Dr. Quick what was the effect of the drugs in producing hypoprothrombinemia?

MAJOR FREDERICK S. COOMBS, JR., Chanute Field, Rantoul, Ill.—I should like to answer Doctor Allen's question, if I may. We have given large intravenous and oral doses of salicylate and have followed the prothrombin time in a number of patients with rheumatic fever. We have found that there is a slight, but definite, increase in the prothrombin time which is proportional to the plasma salicylate level. However, the prothrombin time increased from a normal of 13 seconds to as high as 20.5 seconds when the plasma salicylate level approaches 70 mg. per cent. In a second group of well persons the administration of 6 Gm. of salicylate per day for more than a week did not affect the prothrombin time, either when measured on whole or 12½ per cent plasma.

DR. OVID O. MEYER, Madison, Wis.—We have had a running controversy for two or three years on this matter of the use of dilute or whole plasma in the determination of the prothrombin time. Professor Link uses dilute plasma because he argues that he can detect minor, but significant, changes. In the clinic we felt that if we could not detect change with whole plasma, the changes were not clinically significant. We want as great simplification as possible. In this matter of the use of xanthines in the animal, Link has stated that you get hyperprothrombinemia and, by inference, that clotting may be favored and thus an undesirable effect on the coronary circulation result, rather than a beneficial effect. I think that with careful analysis one would be of the opinion that hyperprothrombinemia, whether it exists or not as a result of xanthine administration, is not of such order that one might fear it in the usual clinical practice. Another problem is that of hemorrhage possibly resulting from the administration of salicylates. I make a particular point of this because again we have some disagreement. Clinical reports to date arguing for the important role of salicylates in the production of hemorrhage are not too impressive. Hypoprothrombinemia produced by salicylate administration to man is ordinarily mild and in itself does not seem to be of a degree to result in hemorrhage.

I have one question for Doctor Quick and that is on the matter of congenital hyperprothrombinemia. I wonder whether or not this may be a matter of thromboplastin abnormality? Prothrombin is a pretty vague substance and, unless you use several types of thromboplastin, might it not be possible that the blood of this patient would have an effect on the thromboplastin in such a way that we would have an abnormal result irrespective of the prothrombin level?

There is another question. I noted that in this student weighing 188 pounds you used 40 mg. of vitamin K. I wonder if when you have heavy individuals you might not have to use 60 or 80 mg. for the desired results?

DR. L. N. KATZ, Chicago, Ill.—I would have wished that Doctor Quick had found that the xanthines had accelerated the prothrombin time. Implicit in the discussion is the implication that these drugs are valuable in the treatment of coronary disease. I simply wish to state that the burden of proof is still upon those who maintain this is so as far as oral xanthines are concerned.

DR. QUICK (closing).—In the studies which I have made on the administration of large doses of vitamin K orally in animals, I have noted no particular change in the clotting time or in the concentration of prothrombin. When one gets abnormal results such as clotting before the blood can be decalcified, it is difficult to offer any satisfactory explanation.

I think that Major Coombs has pretty well answered the question concerning salicylates, and the remarks of Doctor Meyer have also answered it. Recently it has been claimed that quinine causes hypoprothrombinemia. I have repeated those experiments and have been unable to find any decrease in prothrombin after quinine. We are probably going to discover in addition to dicumarol and the salicylates other drugs that will reduce prothrombin. The sulfonamides which suppress the bacterial flora of the intestines can in certain individuals reduce the available vitamin K sufficiently to cause a serious hypoprothrombinemia.

The inquiry concerning thromboplastin brings up the question as to what the ideal reagent for activating prothrombin is. I have found dehydrated rabbit brain consistently satisfactory, and I have reason to believe that it is as close to the natural activator as any other known natural material.

I think that the case of hypoprothrombinemia which I have presented is a true congenital one and that the defect has gone through a number of generations. I think that if we would examine large groups of people we would find an occasional case of primary hypoprothrombinemia. I am not qualified to discuss the use of xanthines in heart disease.

THE EFFECT OF THIOURACIL ON LEUCEMIA WITH A CLINICOPATHOLOGIC REPORT OF A CASE OF CHRONIC MYELOID LEUCEMIA THAT DEVELOPED AN EXTREME NEUTROPENIC LEUCOPENIA

LOUIS R. LIMARZI, M.D., CONRAD L. PIRANI, M.D. (BY INVITATION), AND
RICHARD J. KULASAVAGE, M.D. (BY INVITATION), CHICAGO, ILL.

One of the toxic reactions of therapeutic doses of Thiouracil in the treatment of hyperthyroidism is the production of a granulopenia. Both a nonfatal and fatal type of agranulocytosis following administration of thiouracil have been reported. The selective action of thiouracil on the granulopoietic tissue is the basis for the therapeutic evaluation of the drug in leucemia. Four cases of chronic myeloid leucemia and one case of chronic lymphatic leucemia were treated with the drug.

The total amount of thiouracil given varied from 1.6 Gm., administered in divided doses over a period of eight days, to 274 Gm., given in divided doses over a period of three months. The average daily dose ranged from 0.2 to 3.0 Gm. In three cases of myeloid leucemia and in the case of lymphatic leucemia, the drug was without effect on the leucemic process in either the blood or bone marrow, basal metabolic rate, or blood chemistry. There was no clinical improvement. In one of the cases of myeloid leucemia, with specific cutaneous

lesions that terminated with an acute blood pattern ("hiatus leukemicus"), the hemopoietic, as well as the other, organs showed a general infiltration of tissue by myelogenous cells of all types. There was no morphologic thyroid hyperplasia. In another case of myeloid leucemia, in which a remission was established with roentgen treatment and liquor potassii arsenitis (Fowler's solution), 0.8 Gm. of thiouracil daily for a period of three months failed to maintain this remission or prevent a relapse of the leucemic process.

A fourth case of leucemia in a Negro woman, 50 years of age, with a leucocyte count of 440,000 and a chronic myeloid blood pattern, was maintained on a daily dosage of 3.0 Gm. No other therapy for the leucemia was given. In three months the leucocyte count gradually dropped to 60,000, and the drug was discontinued. The blood pattern was still that of a chronic myeloid leucemia. There was no improvement in the clinical condition nor a decrease in the size of the enlarged spleen. The basal metabolic rate was still elevated, and there were no blood chemical changes. In the five weeks that followed, the total white count gradually dropped to 1,900, with a differential count consisting of 18 per cent myeloblasts, 33 per cent basophiles, and 49 per cent lymphocytes. There was a complete absence of neutrophiles and eosinophiles in the blood. The blood uric acid gradually increased to 11.4 mg. per 100 c.c. of blood. The patient developed toxic disturbances of the central nervous system consisting of convulsive seizures, myoclonic contractions of various muscles, particularly of the facial muscles, and frequent and severe stages of somnolence and confusion. At autopsy the bone marrow showed numerous myeloblasts and lymphocytes and a complete aplasia of mature and immature types of neutrophiles and eosinophiles. All the organs, including the hemopoietic tissue, showed a minimal amount of leucemic infiltration consisting of myeloblasts and a conspicuous lymphocytic reaction. The hemopoietic organs revealed a marked increase in reticulo-endothelial elements. No histologic evidence of a thyroid hyperplasia was observed in biopsy material taken during the administration of thiouracil or at autopsy. Except for a few myeloblasts and lymphocytes in the blood vessels, the brain tissue was free of any leucemic infiltrations. It is concluded that (1) large daily doses (3.0 Gm.) of thiouracil for a period of three months will produce an extreme neutropenic leucopenia with a minimal leucemic infiltration in the organs and a complete aplasia of neutrophilic elements of the bone marrow; (2) thiouracil produces toxic disturbances of the central nervous system; (3) in cases of myeloid and lymphatic leucemia small doses (0.8 Gm. or less daily) of thiouracil for short or long periods of time is without effect on the leucemic state; (4) in cases of chronic myeloid leucemia with a high percentage of basophiles in the peripheral blood, the basophiles are resistant to the toxic effect of thiouracil and in this respect simulate the blood pattern observed in remissions induced by roentgen therapy; (5) there is no clinical improvement in thiouracil-treated cases of leucemia; (6) thiouracil in sufficient doses regularly inhibits granulopoiesis and destroys granulocytes, an effect which is independent of individual susceptibility.

REACTIONS FROM UNIVERSAL DONOR BLOOD

ROBERT C. HARDIN, M.D., IOWA CITY, IOWA

(INTRODUCED BY WILLIS M. FOWLER, M.D.)

It is the established practice in transfusion to use donor blood of the same A-B type as that of the recipient. Unquestionably, this procedure is theoretically more correct than the administration of universal donor blood without regard for the patient's blood group. It is probably also true that the danger incident to the use of universal blood has been exaggerated.

Recent experience in the Army Medical Service affords the opportunity to make certain comparisons between the reaction rates encountered in transfusion of group-specific blood and universal donor blood. Two series of transfusions can be compared; the first is composed of 7,299 transfusions of group-specific blood and, the second, of 9,392 transfusions of universal donor blood. In the first, a reaction rate of 4.22 per cent was encountered and, in the second, a rate of 4.80 per cent. The universal donor blood was not screened for high agglutinin content.

Because the mechanism by which reactions peculiar to universal donor blood arise is the introduction of an agglutinin into a recipient possessing the corresponding agglutigen, one is particularly interested in the frequency of hemolytic reactions. The rates in the two series were equal, being 0.20 per cent and 0.18 per cent, respectively.

Mild hemolytic reactions might easily be classified clinically as pyrogenic. Therefore, the difference of 0.50 per cent in pyrogenic reactions between the two series (3.18 per cent and 3.68 per cent) merits closer scrutiny. The rate of pyrogenic reactions was computed for each of the twenty-three weeks during which the study was made. In the first two weeks the use of universal donor blood was characterized by a high reaction rate (9.09 per cent and 7.72 per cent). Subsequently, this class of reactions was equally frequent in both series. The sharp decrease in pyrogenic reactions is evidence that some other mechanism than intravascular hemolysis operated, since reactions on that basis would tend to maintain their frequency. The fall in reaction rate followed the correction of errors in the technique in preparation of diluent fluids.

Conclusion.—No essential difference in reaction rates in two series of transfusions, one of group-specific blood and the other of universal donor blood, was encountered.

DISCUSSION

DR. C. C. STURGIS, Ann Arbor, Mich.—I would like to ask if there were any fatal reactions in these two groups which could be ascribed to the blood transfusion?

QUESTION.—How do you differentiate between pyrogenic and allergic reactions, and what is the time of the reaction following transfusion?

DR. ROBERT W. HEINLE, Cleveland, Ohio.—I certainly agree that universal donor blood can cause trouble at times, but certainly such reactions are uncommon. When it is difficult to get Rh-negative blood, we use a universal donor. When we do use universal donor blood, we set up a minor cross match with the donor's serum diluted 1:100. If no agglutination results

with 1:100 dilution, we feel that it is safe to go ahead with type O blood because the titer is not sufficient to cause trouble. If the titer is sufficient to show up in a 1:100 dilution, it is evident that we are dealing with an unusually high titer and that the blood should not be used.

DR. HARDIN (closing).—The death rate following transfusions, but not necessarily due to the transfusion, was .06 per cent. The problem of the differential diagnosis of transfusion reactions is one which always causes considerable trouble. Allergic reactions are usually classified as those characterized by urticaria or asthma. Pyrogenic reactions are differentiated from hemolytic reactions by drawing from the patient from 3 to 5 c.c. of blood, centrifuging, and inspecting for free hemoglobin in the plasma. That type of test will not rule out all kinds of hemolytic reactions, particularly the Rh reaction in which the hemolysis may be delayed. The usefulness of the time factor in transfusion reactions is also open to question because sometimes the phenomena of the hemolytic reaction may appear late. However, in the true A-B incompatibility, the symptoms usually appear before the first 50 to 75 c.c. of blood have been administered.

EFFECT OF RESUSPENDED ERYTHROCYTE TRANSFUSION ON SERUM PROTEIN

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The presence of hypoproteinemia is commonly considered a contraindication for resuspended red cell transfusion as a substitute for whole blood.

Total protein determinations were made on patients immediately before and twenty-four hours after resuspended red cell transfusions. In patients who showed a marked anemia and hypoproteinemia, a definite rise in serum protein level generally occurred in twenty-four hours. In patients with hypoproteinemia, but no anemia, no significant change in serum proteins could be demonstrated despite repeated resuspended red cell transfusions sufficient to produce a polycythemia state. Most patients with anemia and serum protein levels within a normal range showed a very slight rise or no change in serum protein level, while a few patients showed a slight drop.

Dogs made hypoproteinemic by plasmapheresis showed no appreciable rise in serum proteins following large repeated red cell transfusions during the period of experiment.

It appears from these studies that the incomplete protein contained in erythrocytes (mainly hemoglobin) is not available in appreciable amounts for conversion into plasma proteins during the period of our observations.

The rapid rise in plasma protein demonstrated in patients with anemia and hypoproteinemia may be explained on the basis of improvement in the metabolism in the liver by correcting the anemia and the protein sparing action caused by relieving the demand for hemoglobin production.

DISCUSSION

DR. DONALD D. KOZOLL, Chicago, Ill.—We were interested in treating patients with hypoproteinemia, without anemia, by the use of red cell suspensions washed twice with dextrose and then rediluted with dextrose. Although we did see an improvement in the total serum protein, it did not occur until we gave more than two liters of red cells.

We further checked the effect of red cell suspension on the albumin-globulin ratio and found that wherever there was a rise in the total serum protein, it occurred in the globulin fraction. Appreciating the greater importance of an improvement in the albumin fraction than the globulin, we have been skeptical of the efficacy of this method of treating protein deficiencies.

DR. WARREN B. COOKSEY, Detroit, Mich.—With the decline of enthusiasm concerning the use of suspended red cells, it is not, therefore, such an important subject. I would not agree to that thought, however, because it seems to me that the increasing use of blood is going to make it necessary for us to conserve blood supply, and that view may well hurt the increasing use of plasma fractions. Certainly there are programs in the air at this time in which the application of albumin-globulin ratios, etc., is a part of postwar planning for blood supply. We, in Michigan, particularly are interested in what the future blood supplying agencies will have. We are thinking very much in terms of salvaging cells. In Detroit some of us connected with the Red Cross had an unusual opportunity with Parke, Davis & Company of this same city to salvage a good many red cells, and we supplied 26,000 pounds of these suspended cells to different hospitals. We had no opportunity to make detailed studies. On the other hand, we did some protein studies and found that in some cases supplied quickly with red blood cells there was a rise. Our hospitals which received these cells varied a great deal. Some were hospitals caring for patients with chronic tuberculosis, some caring for patients with other chronic diseases, as at Eloise, which has a great many patients with cancer. The opinion was that these cells certainly were a most useful adjunct to treatment. The statement was made that the patients had an increased sense of well-being and that they really improved. People who were tired and not able to get out of bed were improved and able to get out of bed quickly with massive transfusions. Whatever the answer is in regard to what happens to the protein portion of the blood, we still feel that transfusions can serve a most useful purpose. It was our very good opportunity to supply it in large quantities, and we had very enthusiastic support.

THE SYNDROME OF HYPERVENTILATION

GEORGE L. ENGEL, M.D., EUGENE B. FERRIS, M.D., CHARLES D. STEVENS, PH.D.

(BY INVITATION), MYRTLE LOGAN, M.D. (BY INVITATION), AND JOSEPH

P. WEBB, M.D. (BY INVITATION), CINCINNATI, OHIO

Involuntary hyperventilation is a not uncommon manifestation. It may occur as a hysterical symptom, as a physiologic concomitant of the emotions of fear or anger, or as the result of diffuse or focal encephalopathy. The symptoms commonly experienced during hyperventilation include numbness and tingling of the extremities and face, buzzing in the head, decreased consciousness, and tetany. Rarely actual syncope occurs.

The mechanism of some of the symptoms has been elucidated in normal subjects by study of the E.E.G.*; arterial and internal jugular venous blood gases, sugar, and pH; and pulse and blood pressure during voluntary hyperventilation. It was found that different individuals differed in the ease with which different symptoms developed, even though the largest part of the change in arterial CO₂ content and pH was attained within a minute, was comparable in all subjects; and changed but slightly thereafter. Reduction in the level of consciousness occurred readily in some individuals and infrequently in other individuals, but in each individual the disturbance in consciousness was greatest (1) with lower blood sugar, (2) with low oxygen tension of the inspired air,

*Electroencephalograph.

and (3) in the upright position; conversely high blood sugar, high oxygen tension, and the recumbent position protected consciousness. In all instances, the reduction in consciousness correlated with the degree of slowing of the E.E.G.; with marked slowing, unconsciousness occurred; with slight slowing, there was no demonstrable reduction in the level of awareness. When consciousness became markedly reduced, the subject automatically stopped overbreathing and did not start again until E.E.G. slow waves had disappeared.

Study of the O_2 , CO_2 , glucose content, of pH of the internal jugular blood, and of arteriovenous differences failed to reveal any correlation with E.E.G. slowing or changes in consciousness. This is contrary to the findings of Gibbs, Gibbs, Lennox and Nims.* It was discovered that blood taken simultaneously from both internal jugular veins followed two entirely different patterns in more than one-half of the cases. When the changes in both jugular veins were the same, either pattern could be found. Pattern I included: (1) marked drop in O_2 content, (2) slight drop in CO_2 content, (3) slight rise in pH, and (4) marked drop in sugar content. Pattern II included (1) slight fall in O_2 content, (2) marked fall in CO_2 content, (3) moderate rise in pH, and (4) slight fall in sugar content. These differences are thought to result from individual anatomical variations in the venous contributions to the left and right internal jugular veins, respectively, and account for the failure to correlate the electrical activity of the cortex with the chemical changes. Different portions of the intracranial contents apparently behave differently in regard to venous drainage, blood flow, and metabolism, and it is difficult to evaluate the contribution of the cerebral cortex to internal jugular venous blood in any instance.

Tetany and the subjective symptoms of numbness and tingling were found to occur after longer periods of hyperventilation and, hence, were more common and severe under circumstances where consciousness was retained. In general, if slow E.E.G. waves did not appear within three minutes of vigorous hyperventilation, they were unlikely to appear, while tetanic phenomena became more prominent. In prolonged mild hyperventilation, tetany was common, whereas disturbances in consciousness were unusual. In such instances, hyperventilation was usually involuntarily terminated by muscular fatigue and by tetany of the respiratory muscles.

Actual syncope, with unconsciousness and falling, occurred only in very anxious persons in whom the unpleasant symptoms led to typical vasodepressor syncope, in hysterical patients who also had hysterical syncope, and in rare individuals with poor postural adaptation. In normal individuals blood pressure and pulse showed only slight change in the recumbent position and but moderate narrowing of the pulse pressure and tachycardia in the erect posture.

Syncope due to peripheral circulatory collapse is not a normal component of the hyperventilation syndrome.

DISCUSSION

DR. EDWARD H. RYNEARSON, Rochester, Minn.—I was much interested in your comments on hyperventilation. I think many physicians have had patients whose condition was at first

*Arch. Neurol. & Psychiat. 47: 879, 1942.

considered to be hyperinsulinism but later discovered to be hyperventilation. In one such case that I recall, the level of blood sugar was 40 mg. per 100 c.c. I would like to ask what the lowest blood sugar is that you have encountered.

Two statements can be made about this matter: first, that hyperventilation often can be incorrectly diagnosed as hyperinsulinism; second, that I can remember no instance in which true hyperinsulinism was diagnosed as hyperventilation.

DR. C. C. STURGIS, Ann Arbor, Mich.—I observed a patient with hyperinsulinism who developed hyperventilation under excitement, with the classical changes of tetany in the hands.

DR. GEORGE E. WAKERLIN, Chicago, Ill.—I should like to hear Doctor Engel discuss the relation of blood pressure changes in his subjects to the hyperventilation syndrome. Previous studies have indicated that hyperventilation produces a hypocapnia with a resulting decrease in the tonus of the vasomotor center consequent upon a central action and reflex effect of the hypocapnia upon the vasomotor center. The resultant lowering of arterial blood pressure has been previously considered as playing an important role in the production of altered cerebral cortical activity in hyperventilation.

DR. FORD K. HICK, Chicago, Ill.—Can normal subjects continue hyperventilating for very long? I wonder what would be the effect of a number of cumulative and severe episodes of hyperventilation.

DR. ENGEL (closing).—In answer to the question about blood sugar, the blood sugar levels were determined in internal jugular venous blood. When blood from the arm vein is used, one finds very little change in the blood sugar level. The blood sugar level in the arterial blood may rise, or fall slightly, so actually total hypoglycemia is not an important phenomenon, although it would seem that sugar may be used more rapidly by the brain.

In regard to hyperventilation during hypoglycemia, I think that would occur occasionally because a patient experiencing mild disturbances in consciousness, such as one sees in hypoglycemia, becomes anxious, and anxious people are likely to hyperventilate.

In regard to changes in blood pressure in the different postures, we studied blood pressure in all patients, and there was no correlation between changes in blood pressure and disturbances in the level of consciousness. In the recumbent position the blood pressure is likely to change very little, and disturbances in consciousness may be marked in the recumbent position. In the erect position, there may be some fall of blood pressure and some narrowing of pulse pressure but not to a marked degree. Some patients when first experiencing these distressing symptoms may become frightened and develop vasodepressor syncope. In these instances, one sees a period for a minute or two after hyperventilation has stopped during which blood pressure is maintained and then the development of typical vasodepressor syncope with falling blood pressure and loss of consciousness if the patient is erect. During the marked disturbance in consciousness with hyperventilation, the patient usually does not fall. That is one of the reasons why the disturbance in consciousness has not been as apparent to most observers as we have described it.

The length of time the patient can hyperventilate is limited by three things: (1) loss of consciousness, during which the patient usually stops hyperventilating involuntarily; (2) tetany, which inhibits the action of the respiratory muscles; (3) fatigue. We usually allowed from a ten- to twenty-minute rest period between experiments when we carried out more than one experiment on the same subject. I do not think this accounts for the differences we observed, because we varied the order of experiments in most instances.

METABOLIC AND PHYSICAL CHANGES ASSOCIATED WITH THE
ORAL AND INTRAVENOUS ADMINISTRATION OF AMINO
ACID PREPARATIONS TO MAN

CHARLEY J. SMYTH, M.D., CARL A. MOYER, M.D. (BY INVITATION), AND
ANDREW G. LASICHAK, M.D. (BY INVITATION), ELOISE, MICH.

This study was conducted upon eight active male adults. Each subject was offered a standard 3,544 calorie diet. Following control periods on this diet, amino acid solutions* were given as supplementary nitrogenous food either orally and/or intravenously (equivalent to 100 Gm. of protein per day) in 480 c.c. of sterile water three times daily. The rate of infusion varied from 10 to 40 Gm. per hour.

The following determinations were made: urinary volume, pH, total nitrogen, ammonia nitrogen, chlorides and creatinine; blood chlorides, CO₂ combining capacity, and hematocrit; and body weight. The only consistent changes observed during the administration of amino acid preparations I and II were an elevation of total nitrogen and ammonia nitrogen excretions.

The subjective changes noted during the injection periods were dizziness, nausea, vomiting, anorexia, and weakness. The severity of these was directly related to the rate of administration. However, relatively severe symptoms were noted with relatively slow rates of injection (15 Gm. in two hours) of preparation II.

The objective changes during and subsequent to the injection periods were listlessness, apathy, irritability, and dependent edema. There was no apparent correlation between the subjective and objective observations and blood and urine chemical changes.

DISCUSSION

DR. W. S. HOFFMAN, Chicago, ILL.—It would be very unfortunate if one gained the impression that distressing symptoms are likely to follow the injection of any of the available commercial preparations of amino acids. At the Cook County Hospital we have acquired a great deal of experience during the last two years with parenterally injected amino acid, particularly with Parenamine. We have made injections into hundreds of patients. In many cases we have given as much as 135 Gm. of amino acids a day for as long as three weeks. Very seldom have our patients experienced any untoward symptoms with the injection, and then only when the injections were made at rates higher than 300 c.c. of a 45 per cent solution per hour, or when the patient had a hepatitis. We have data to be published which indicate that if the plasma amino acid nitrogen levels rise well above 10 mg. per 100 c.c., nausea and vomiting may appear, but usually not otherwise.

In the early enthusiasm for the use of parenterally injected amino acids, the administrations were made too rapidly and in too great a quantity in an attempt to build up patients with protein deficiency to normal in as fast a time as possible. We know now that in patients with severe protein deficiency in which as high as from 450 to 500 Gm. of nitrogen had been lost, it is futile to expect a restoration to normal by parenterally injected amino acids alone. The best that can be accomplished is a slight positive nitrogen balance until these injections can be supplemented with orally administered protein. Under such circumstances, 60, 90, or, at the most, 120 Gm. of amino acids should be given per day, at a rate of 15 Gm. per hour. There should be no difficulty, then, especially if hypertonic solution can be avoided.

*Preparation I "Amigen," Mead Johnson and Company, Evansville, Ind.

Preparation II "Parenamine," Frederick Stearns & Company, Detroit, Mich.

We have made a study of blood chemical changes during and after intravenous injections of amino acids which we are about to publish, and we can confirm Doctor Smyth's findings that the changes are insignificant, except for the rise in plasma amino acid nitrogen. One of the unusual findings was a drop in inorganic phosphate comparable to what one sees after glucose injection.

DR. G. E. WAKERLIN, Chicago, Ill.—As I understand it, Amigen is an enzymic casein digest containing 70 per cent amino acids and 30 per cent peptides, whereas Parenamine is an acid hydrolysate consisting entirely of amino acids. Does Doctor Smyth have any explanation for the depression of appetite produced by Parenamine in contrast to Amigen? In view of Doctor Hoffman's comment, the higher amino acid content of Parenamine may be a factor.

DR. SMYTH (closing).—I think it is well to point out that the rates we have used have been two or three times as great as advocated by commercial houses. Our interest in the problem arose when it was observed that patients did not eat the meal following the administration of amino acid mixtures.

With reference to Doctor Wakerlin's question, I have no answer as to why one mixture depresses the appetite more than the other. We are at present investigating a mixture of the ten essential amino acids commercially prepared.* The effect of fortifying this mixture with aspartic and glutamic acid is being studied. Madden and Whipple have shown that these two amino acids are the cause of gastrointestinal symptoms in dogs.

PHOSPHOLIPIDS OF LIVER AND BLOOD STUDIED WITH RADIOACTIVE PHOSPHORUS

JESSE L. BOLLMAN, M.D., AND EUNICE V. FLOCK, PH.D. (By Invitation)
ROCHESTER, MINN.

After a single administration of radioactive phosphorus as disodium phosphate to rats, this substance rapidly enters the liver and furnishes a label for the inorganic phosphate of the liver. The radioactivity of the phospholipid of the liver gradually approaches that of the inorganic phosphate of the liver. Calculations based on the exchange of radioactive phosphate from the inorganic fraction to the phospholipid, from analysis made at intervals from thirty minutes to eighteen hours, indicate that approximately 3.5 mg. of P forms new phospholipid in 100 Gm. of liver each hour. The total amount of phospholipid P, from 120 to 140 mg., remains unchanged, so that an equal amount leaves the liver or is metabolized. Approximately 0.8 mg. enters 100 c.c. plasma each hour as phospholipid, and an equal amount leaves the plasma, the normal value of from 3.5 to 4.5 mg. remaining unchanged. These figures account for enough phospholipid turnover in the liver and plasma to metabolize or transfer fat equivalent to only 3 per cent of the calorie needs of the animals and indicate that phospholipid formation is probably not an obligatory step in fat oxidation or transfer.

Rats receiving diets widely divergent in protein, carbohydrate, and fat content may show corresponding divergence in the concentration of phospholipid in the liver, however, if the altered size of the liver is considered, it is found that the formation of new phospholipid for each whole liver is very similar. A deficiency of dietary choline does not greatly reduce the amount of new phospholipid formation, but administration of choline will increase new phospholipid formation. Damage to the liver by carbon tetrachloride does not reduce phospholipid formation. Choline administration may increase phospholipid formation after injury to the liver.

*By Merck & Company, Inc., New York, N. Y.

SEQUELAE OF TRENCH FOOT AND THEIR TREATMENT BY LUMBAR SYMPATHECTOMY

MAJOR DAVID I. ABRAMSON AND LIEUTENANT COLONEL HARRIS B. SHUMACKER, JR.
(BY INVITATION), GALESBURG, ILL.

The clinical picture of trench foot, from four to thirteen months after exposure, was studied in a series of 567 patients. The sequelae were divided into three categories.

In the first group, the findings were primarily the result of excessive sympathetic tonus. They consisted of a low cutaneous temperature of the toes, hyperhydrosis, and cyanosis, which at times was replaced by rubor, mottling, and a subjective sensation of coldness of the feet on exposure to a cool environment.

In two patients in whom hyperhydrosis was so marked that maceration of the skin followed by secondary infection resulted, sympathectomy produced an immediate therapeutic effect. The infection cleared up shortly after the cessation of sweating. The operation was also performed in two individuals with cold, blue, wet feet, who complained of pain on exposure to a cold environment. The feet became red and dry, and the marked responsiveness to cold was no longer present.

In the second group, the findings were suggestive of some type of peripheral nerve involvement. They consisted of tenderness of the sole of the foot, resulting in abnormal gaits, hypesthesia, paresthesia, and numbness.

In ten patients of this group, sympathectomy was done in order to treat the plantar tenderness. In six of these, little alteration in symptoms was produced, while in four, slight or moderate relief of pain was noted. Such complaints as paresthesia, hypesthesia, and numbness were unaffected.

The third group showed signs both of excessive sympathetic tone and peripheral nerve involvement. Besides these, desquamation of the plantar surface of the foot, swelling and stiffness of the toes, osteoporosis, and atrophy of the small muscles of the feet were fairly common findings. Sympathectomy was not attempted in the treatment of any of these manifestations.

In the case of gangrene of toes or a portion of the foot, sympathectomy was performed as a preliminary step in twenty-six patients.

It is concluded that this procedure is indicated in the treatment of hyperhydrosis complicated by secondary infection and infrequently in the case of the patient who is incapacitated by a cold, wet, cyanotic foot which is markedly responsive to a cold environment. It also appears to increase the rate of healing of the stump after removal of gangrenous material. Only on occasion does it have any therapeutic effect on the neuritic symptoms of trench foot.

DISCUSSION

DR. GLAZ DE TAKATS, Chicago, Ill.—One is beginning to see trench foot in civilian practice and in the veterans' facilities. I fully agree with Major Abramson that the vasomotor group is benefited by sympathectomy, and obviously patients with dysesthesia of the feet will not respond. Nevertheless, occasionally a patient will present himself whose burning pain resembles the causalgic state and in whom sympathetic block abolishes the burning paresthesia.

Such patients are also seen in air hammer disease and unquestionably suffer from demyelination of some of the sensory fibers. Sympathectomy in such a patient will not relieve the true sensory defect but seems to abolish the sensory stimulation initiated by sympathetic fibers in the demyelinated segments. My experience with a considerable number of cases at the Veterans Hospital would indicate that the majority of patients suffering from trench foot do not require sympathectomy.

DR. JOSEPH EDWARDS, St. Louis, Mo.—In our experience in Italy in 1943 and 1944 at the Twenty-first General Hospital, we had about 350 patients with trench foot. Because of the severity of these cases, it was thought wise to do sympathectomy in a certain selected group. We obtained permission of the theater consultant surgeon, Colonel Churchill, for this work. Accordingly, unilateral sympathectomy was done by Lieutenant Colonels H. G. Schwartz and F. A. Simone, on thirteen patients most of whom had causalgia, coldness of the feet, and cyanosis. Our conclusions were the same as Major Abramson and Lieutenant Harris B. Shumacker's, that in certain selected patients it seems to be beneficial. We thought only of very severe cold injury with hyperhydrosis often complicated by infection. In some cases of early gangrene, if seen early enough, there may be benefit from sympathectomy. It was of definite benefit in patients with epidermophytosis. We reported some of this work in the United States Army Medical Bulletin. We had occasion to follow these patients four months only. We hope to follow this work by going over the records of these men after they return to the states.

MAJOR ABRAMSON (closing).—With respect to Doctor de Takats' discussion, I cannot add anything further to what I have already stated. There is only an occasional individual with trench foot in whom sympathectomy is followed by a loss of the symptoms of nerve irritation. It is of interest that two of the men who demonstrated marked sensitivity of the soles of the feet also had similar complaints in the heels, and it was the symptoms in the heels which disappeared following operation.

In response to Doctor Edwards' statements, we have seen patients in whom sympathectomy had been performed five, six, or seven months before coming to our hospital, and little difference was noted between the denervated limb and the control with respect to the severity of the response to changes in environment. In fact, some of these patients complained more in the sympathectomized extremity than in the normally innervated one, but the explanation may be that the sympathectomized extremity was the worse one originally.

TOXIC MANIFESTATIONS FOLLOWING LARGE DOSES OF ERTRON

SMITH FREEMAN, M.D. (By Invitation), PAUL S. RHOADS, M.D., AND
LEONA B. YAEGER, M.D. (By Invitation), CHICAGO, ILL.

Two case histories are presented in which the following findings were similar: ingestion of large doses of Ertron and high milk consumption over long periods followed by extreme weakness, restlessness, subnormal body temperature, weight loss, anorexia, generalized aching, dermatitis, inflammation, and small fatty deposits of the sclerae. Both patients had disturbed calcium metabolism, increased nitrogen retention in the blood, reduced renal function, anemia with eosinophilia, albumin, and casts in the urine. In one patient there was extensive calcification in many bursae and exfoliative dermatitis.

Many of these findings are known to occur in animals poisoned with vitamin D.

In view of the large numbers of persons now being treated with ertron and other high potency vitamin D preparations, a careful check on the complete therapeutic history of patients with the mentioned symptoms is in order. Also

all physicians treating patients with large doses of ertron and other high potency vitamin D preparations should keep a careful check on the urinary findings and calcium-phosphorus value of the blood in addition to insisting on a low calcium diet.

DISCUSSION

DR. ELMER SEVRINGHAUS, Madison, Wis.—This syndrome from the administration of large doses of vitamin D is not limited to ertron and it is not limited to the treatment of chronic arthritis. I doubt that it is caused by large intakes of milk. Two cases were reported from the New Haven Hospital, and I have had one case. Large doses of vitamin D are very helpful in tetany. I have used doses of the magnitude reported without encountering any difficulty. Recently, I have seen one such patient under treatment with 100,000 units of Viosterol who developed the same subjective picture, except that there were no skin manifestations. There were anemia and reduced renal function, clouding of the consciousness, and a peculiar nervousness. Withdrawal of vitamin D with the liberal use of saline administered intravenously brought the serum calcium down from 17 mg. per 100 c.c. of blood, which is a dangerous level. I should say that doses of 50,000 units of vitamin D and upward might be followed by toxic symptoms.

DR. FREEMAN (closing).—It has been shown, for example, that a transient hypercalcemia may be produced by calcium taken orally; however, a more marked and prolonged hypercalcemia will result from the same amount of ingested calcium if the animal has previously received large doses of vitamin D. It has never been possible to dissociate the toxic effects of vitamin D from its influence upon calcium metabolism. For three or four years, I have followed the serum calcium values in several patients with hypoparathyroidism receiving large doses of vitamin D. Such patients may go along all right for months on a given dose of vitamin D and a certain calcium intake, and then for no apparent reason they may develop a hypercalcemia accompanied by symptoms of toxicity. This may be due to an accumulative effect from vitamin D. The prolonged action of vitamin D has been shown in the treatment of rickets and osteomalacia as well as by toxicity studies. Large doses of vitamin D may influence calcium metabolism for months after the vitamin has been discontinued. It is not possible to predict from one patient to the next what dose of sterol will be required to correct a hypocalcemia of a certain magnitude. The requirements of each patient must be determined individually. There is no substitute for serum calcium determinations, and these should always be done in duplicate.

Both the clinical and experimental evidence indicates that a high calcium intake increases the likelihood of toxic manifestations from large doses of vitamin D. Conversely, proper selection of diet and calcium intake will minimize the vitamin D required to correct a hypocalcemia.

THE EFFECT OF INFECTION AND TRAUMA ON THE EXCRETION OF URINARY CORTIN*

R. A. SHIPLEY, M.D., AND R. I. DORFMAN, PH.D., CLEVELAND, OHIO

Urinary cortin may be satisfactorily extracted with ethylene dichloride and partially purified by the extraction of inactive material with sodium hydroxide. A cold test modified from Selye and Schenker and a liver glycogen test as used by Dobriner and co-workers are both sufficiently sensitive for the quantitative estimation of urinary cortin. Groups of animals employed in the cold test must be controlled for sensitivity by including a group which receives a standard

*Work done under contract with the Office of Scientific Research and Development.

dose of active adrenal cortical material as well as a group which receives only the solvent. The cold test is potentially four times as sensitive as the glycogen test and thus requires less urine for an assay. However, satisfactory cold test assays may be obtained in only two of three attempts due to variations in sensitivity of animals as a group. Results are more reproducible with the glycogen test. Both tests (assuming the use only of highly sensitive animals in the case of the cold test) give errors of estimate within the range usually encountered in bio-assay procedures. In the case of the glycogen test the error factor (with ten animals in a group receiving the unknown, and $p = 0.05$) is 1.6. This means that the chances are one in twenty that the true value will not vary by more than 1.6 or $1/1.6$ times the observed result.

In a series of nineteen normal young men and women the daily excretion of urinary cortin as estimated by the cold test averaged 1.1 mg. of compound A equivalent and varied from 0.5 to 1.8 mg. The glycogen test gave values ranging from less than 0.2 to 0.8 mg. There was no obvious difference between men and women. A sample of pooled normal male urine contained 2.0 mg. compound A equivalent by the cold test and 0.4 mg. by the glycogen test. The disparity between values obtained by the two tests might be attributable to the presence in urine of cortical compounds less active in carbohydrate metabolism than against exposure to cold.

Eighty patients with infectious disease were studied, two after herniorrhaphy and three after extensive body burns. In all three categories there was usually a rise in the output of cortin. The augmentation was high as eightfold in some instances. A patient with a clinical diagnosis of Waterhouse-Friderichsen syndrome excreted appreciable amounts of active material. The postoperative rise occurred during the first two days after operation. During recovery from the mentioned types of stress, there were no instances of an abnormal depression in output which one would expect in the presence of adrenal exhaustion.

THE EFFECT OF DIET ON THE GROWTH AND SURVIVAL OF ADRENALECTOMIZED RATS TREATED WITH DESOXY- CORTICOSTERONE ACETATE PELLETS*

ALBERT SEGALOFF, M.D., NEW ORLEANS, LA.

(INTRODUCED BY THOMAS FINDLEY, M.D.)

Immature, adrenalectomized, male rats with subcutaneous pellets of desoxycorticosterone acetate were fed various diets. None of the diets contained any carbohydrate, but all had adequate amounts of the vitamins required for growth in the rat (thiamine chloride, riboflavin, calcium pantothenate, nicotinic acid, pyridoxine, choline chloride, α -tocopherol, vitamin A, and vitamin D₃) and U.S.P. XII salt mixture. When the diet contained crude casein and a fatty acid mixture in ad¹ the vitamins and salts, all the animals survived and grew for eight w¹ time the pellets were removed. However, if the fatty

*This
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acids were omitted, or vitamin-free casein was substituted for the crude casein, then the animals neither survived very long nor grew. A total of seventy-eight rats were employed on the four diets.

These preliminary studies indicate that (1) dietary carbohydrate is not essential for the growth and survival of adrenalectomized rats maintained on desoxycholesterone acetate; (2) dietary fatty acids either burn as carbohydrate or permit the utilization of protein by such rats; and (3) there is some substance different from the vitamins required for growth in the rat which is present in crude casein and is required by our adrenalectomized rats.

DISCUSSION

DR. H. T. RICKETTS, Chicago, Ill.—I should like to ask whether any determination of liver function was made on these animals.

DR. SEGALOFF (closing).—We were not able to do liver function tests. The blood sugars were very low in the animals that died and normal in the animals that survived.

CHANGES IN THE PROMINENCE OF THE EYES IN VARIOUS THYROID STATES

BROWN M. DOBYNS, M.D. (BY INVITATION), AND S. F. HAINES, M.D.
ROCHESTER, MINN.

Repeated exophthalmometer measurements of the eyes were made in various types of thyroid disease during the course of treatment.

Thyroidectomy in patients with exophthalmic goiter was usually followed by increased prominence of the eyes. If hypothyroidism supervened, the prominence of the eyes usually increased still further. Treatment of postoperative myxedema usually resulted in regression of the prominence of the eyes to degrees that existed before myxedema developed. Treatment of spontaneous myxedema also resulted in lessening of the prominence of the eyes. Patients with exophthalmic goiter who were treated with thiouracil showed an increase in prominence of the eyes simultaneous with the drop in basal metabolic rate. In most of these instances, the change in the position of the globe was so slight that it could not be detected without measurements. In one patient with severe exophthalmos, the protrusion of the eyes and edema of the lids and of the conjunctivae reached serious proportions during the course of treatment with thiouracil.

DISCUSSION

DR. E. L. DEGOWIN, Iowa City, Iowa.—I should like to reinforce the conclusions of the speakers by reporting the case of a patient whom I recently had a chance to study. This woman is now 61 years old. In 1942 she consulted her local physician because of symptoms which led him to make a diagnosis of myxedema. The laboratory work and basal metabolic rate determinations were very well done. She had all the clinical signs of myxedema, and I think there is no question that she had the condition at that time. Her attending physician started administering thyroid extract; she made a prompt recovery from the symptoms of myxedema and the basal metabolic rate was restored to normal. She has been on thyroid extract in sufficient doses ever since. About six months after beginning therapy with thyroid extract, the doctor noticed that she was developing exophthalmos. This exophthalmos

dose of active adrenal cortical material as well as a group which receives only the solvent. The cold test is potentially four times as sensitive as the glycogen test and thus requires less urine for an assay. However, satisfactory cold test assays may be obtained in only two of three attempts due to variations in sensitivity of animals as a group. Results are more reproducible with the glycogen test. Both tests (assuming the use only of highly sensitive animals in the case of the cold test) give errors of estimate within the range usually encountered in bio-assay procedures. In the case of the glycogen test the error factor (with ten animals in a group receiving the unknown, and $p = 0.05$) is 1.6. This means that the chances are one in twenty that the true value will not vary by more than 1.6 or $1/1.6$ times the observed result.

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*This work was done in the Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tenn.

and varying degrees of toxicity has given us an unusual opportunity to investigate this problem. These data indicated that the cells of all types of nodular or adenomatous thyroid tissue of nontoxic nodular goiter, toxic nodular goiter, exophthalmic goiter, and so-called fetal adenoma studied are functionally autonomous and consistently showed less avidity for iodine and produced less thyroxine and diiodotyrosine than the groups of thyroid cells in the surrounding thyroid tissue. None could be considered so-called hyperfunctioning adenomas.

The diffuse goitrous tissue of two patients with nontoxic diffuse colloid goiter showed great avidity for iodine, but its ability to produce thyroxine and diiodotyrosine did not appear greatly increased. The diffuse hyperplastic tissue of a patient with exophthalmic goiter showed the greatest avidity for iodine and the greatest ability to produce diiodotyrosine and thyroxine.

The paranodular tissue (that is the thyroid tissue surrounding the nodules) of three patients with nontoxic nodular goiter, one having a mild hypothyroidism, acted biochemically to a greater extent like the tissue of nontoxic colloid goiter. The paranodular tissue of a patient with toxic nodular goiter acted biochemically to a greater extent like the tissue of exophthalmic goiter.

THE EFFECT OF SALICYLATE ON THE SEDIMENTATION RATE, FEVER, AND OCCURRENCE OF VALVULAR HEART DISEASE IN ACUTE RHEUMATIC FEVER

MAJOR HARRY A. WARREN (BY INVITATION), LIEUTENANT COLONEL C. S. HIGLEY,
AND MAJOR F. S. COOMBS, JR., MADISON, WIS.

Sodium salicylate has been used in the treatment of acute rheumatic fever for many years. In 1943 Coburn reported a series of thirty-eight patients with acute rheumatic fever, treated with from 10 to 14 Gm. of salicylate daily, in whom valvular heart disease did not develop. Plasma salicylate levels in these patients were found to range above 35 mg. per 100 c.c. In another series Coburn gave smaller amounts of salicylates and obtained lesser plasma values. In twenty-one of sixty-three of these patients, valvular heart disease occurred. We have been interested in the efficacy of salicylate in the treatment of acute rheumatic fever since publication of Coburn's work.

From November, 1942, to September, 1945, we have studied 186 patients with rheumatic fever. Fifty of these patients were given from 10 to 16 Gm. of salicylate daily, orally, until the sedimentation rate remained normal; another forty-eight were given from 10 to 20 Gm. intravenously daily, for seven days, followed by from 10 to 16 Gm. daily, orally; and eighty-eight patients received less than 50 Gm. per week during the acute phase only. Oral or intravenous dosage of 10 Gm. or more per day gave plasma levels in the therapeutic range advocated by Coburn.

continued to progress until the present time without any signs of thyrotoxicosis. Simultaneously with the development of the exophthalmos, she developed localized areas of solid nonpitting edema which many dermatologists, and particularly Dr. Paul O'Leary of the Mayo Clinic, have described in association with exophthalmic goiter. When she came to the hospital, the paradox of a patient with marked exophthalmos and known myxedema intrigued us, and we immediately began to investigate the pituitary. She was then beginning to have a loss of the lower half of both visual fields, and x-ray examination of the skull showed a sclerotic process in the sphenoid bone involving the anterior clinoid process which the radiologist considered most likely to be due to a tumor in that vicinity. Here is a patient who has a tumor in the region of the anterior lobe of the pituitary gland, who first developed signs of thyroid deficiency, and who has been known to develop exophthalmos while receiving adequate doses of thyroid extract. I certainly think that this also brings into question the hypothesis which apparently has been accepted, that malignant exophthalmos is due to the overproduction of thyrotropic hormones as a result of insufficient thyroid hormone.

DR. E. PERRY McCULLAGH, Cleveland, Ohio.—In observing large numbers of patients with exophthalmic goiter over a period of many years and in making attempts to estimate the factor which may be influencing the exophthalmos, I have been particularly impressed with the great difficulty of establishing any type of control for comparison. Exophthalmos may appear in apparent complete dissociation from evidences of overactivity of the thyroid. For instance, it may make its appearance several years before hyperthyroidism is present, and we have seen it appear and become severe for as long as fourteen years after the disappearance of hyperthyroidism and in the absence of either hyper- or hypometabolism. In view of such facts as these, it is a matter of the greatest difficulty to evaluate the effects of any treatment which is given in an attempt to influence exophthalmos.

There is, I believe, considerable reason to doubt the validity of the current belief that thyrotropic hormone is the cause of progressive exophthalmos. If thyrotropic hormone were actually the cause of exophthalmos, it would seem reasonable to expect that exophthalmos would be present in cases of acromegaly with hyperthyroidism. In such patients, there is a pituitary tumor and goiter with hyperthyroidism, and it seems entirely reasonable to suspect that an excess of thyrotropic hormone must be present, and yet no exophthalmos occurs under these circumstances. Patients with spontaneous myxedema also have an excess of thyrotropic hormone, and these patients do not develop exophthalmos. During thiouracil therapy, there is almost certainly a marked increase in thyrotropic hormone production, and as far as an overall experience with thiouracil has been concerned, there is little indication that exophthalmos usually increases during such treatment. In short, it appears that the exophthalmos of Graves' disease is independent of all the influences which we know of including the effect of thyroid. It is quite evident that hypothyroidism itself does not cure exophthalmos, and it seems to me from this evidence alone that it does not seem reasonable to suppose that the feeding of thyroid would be of much benefit. It seems possible that a more adequate means of inhibiting the pituitary than has been used up to the present may be shown to be the most efficient treatment.

THE CLINICAL SIGNIFICANCE OF THE FUNCTIONAL BEHAVIOR OF ADENOMAS OF THE THYROID GLAND

I. DARIN PUPPEL, M.D., CHARLES P. LEBLOND, M.D. (BY INVITATION), ELSIE RILEY, M.S. (BY INVITATION), AND GEORGE M. CURTIS, M.D., COLUMBUS, OHIO

The exact function of the thyroid nodule or adenoma has not been completely established for the reason that there has been no adequate method to determine the functional activity of various parts of a nodular goiter.

Recently, radio-iodine fractionation study of many forms of pathologic thyroid tissue removed from seven patients with varied clinical types of goiter

and varying degrees of toxicity has given us an unusual opportunity to investigate this problem. These data indicated that the cells of all types of nodular or adenomatous thyroid tissue of nontoxic nodular goiter, toxic nodular goiter, exophthalmic goiter, and so-called fetal adenoma studied are functionally autonomous and consistently showed less avidity for iodine and produced less thyroxine and diiodotyrosine than the groups of thyroid cells in the surrounding thyroid tissue. None could be considered so-called hyperfunctioning adenomas.

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We have arbitrarily divided our cases into two groups, severe or moderate, depending upon the initial sedimentation rate. Above 60 mm. per hour was considered evidence of severe disease, while below that figure was considered moderate.

One hundred twenty-six cases fell in the severe category. Forty-five of these patients received the large oral dose of salicylate and required an average of 51.3 days for the sedimentation rate to reach the normal level; forty-four patients received intravenous and then oral salicylate in large doses and required an average of 61.4 days for the sedimentation rate to reach normal; and thirty-seven patients receiving the small oral dosage required 58.7 days for the sedimentation to become normal.

Of the sixty patients in the moderate group, five receiving large oral doses of salicylate required an average of thirty-six days for the sedimentation rate to become normal; four given intravenous and then oral salicylate required an average of 27.75 days for the sedimentation rate to become normal; and fifty-one given small doses of salicylate orally required 35.2 days for the test to become normal.

When all cases are considered, eighty-eight patients receiving small doses of salicylate required an average of forty-five days to reach a normal sedimentation rate. Fifty patients receiving large oral doses required an average of 49.8 days, and forty-eight patients receiving intravenous sodium salicylate required 58.6 days to reach a normal sedimentation rate. When all patients receiving large doses are considered, the average time to reach a normal sedimentation rate was 54.1 days.

Eighty-two patients with a febrile response above 99.2° F. received small doses and required an average of 11.6 days to maintain a normal temperature curve. Eighty-nine patients receiving large doses, either by mouth or by vein, required an average of 4.16 days to maintain normal temperature.

Thirty-five cases, or 39.8 per cent, of those receiving small doses showed a rise in sedimentation rate after reaching a normal level, either with or without clinical signs and symptoms of recurrence. Of those receiving large doses, 61.3 per cent showed such polycyclic courses.

Attempts to determine the presence of organic heart disease were difficult because of the short period of observation. However, seven patients in each group developed significant cardiac murmurs not present on admission or showed definite progressive changes in cardiac abnormalities present on admission.

From these findings, it is apparent that the administration of large doses of salicylates does not reduce the time necessary to reach a normal sedimentation rate and does not protect against the occurrence of polycyclic rheumatic fever. It does eliminate the temperature elevation sooner than small doses. It does not prevent the occurrence of valvular heart disease or prevent the progression of pre-existing cardiac damage to any greater extent than small doses.

DISCUSSION

DR. M. J. SHAPIRO, Minneapolis, Minn.—This report, together with other recent work, shows what has been known for many years: namely, that the salicylates have a good

clinical but no specific effect on rheumatic fever, that the salicylates are rapidly absorbed from the stomach, and that intravenous salicylate therapy is unnecessary and dangerous. Soon after Coburn reported on the use of large doses of sodium salicylate given intravenously, a number of deaths resulted from such therapy.

A word should be said about the use of the sedimentation rate in rheumatic fever as well as about its general use. Not infrequently patients who have apparently recovered from an attack of rheumatic fever continue to have an increased sedimentation rate for weeks and months after all other evidence of activity has disappeared. Such patients are often kept in bed for months for no other reason. Many patients, especially children, have an upper respiratory infection accompanied by joint pains. Such children are frequently kept in bed for long periods of time only because the sedimentation rate is moderately increased. It must be true that many such patients who probably never had rheumatic fever are among those who have been treated prophylactically with the sulfonamides.

If a few of Major Warren's patients were among those who continued to have increased sedimentation rates even though they had recovered from an attack of rheumatic fever, the results of his study would be entirely different.

It is time that the whole matter of the sedimentation rate be restudied. It is quite evident that the increased sedimentation rate alone should not be used as a sole criterion for determining when a patient has recovered from an attack of rheumatic fever.

PENICILLIN AND DICUMAROL, AND PENICILLIN IN THE TREATMENT OF SUBACUTE BACTERIAL ENDOCARDITIS

OVID O. MEYER, M.D., AND CHARLES J. THILL, M.D. (BY INVITATION)
MADISON, WIS.

In June of 1944, it was widely held that penicillin was not efficacious in the treatment of subacute bacterial endocarditis. In January of that year, however, Loewe and his associates reported success in the treatment of this condition with combined penicillin and heparin administration. When penicillin became readily available, in May of 1944, we decided to treat bacterial endocarditis with penicillin and the anticoagulant Dicumarol, a potent cheap drug which can be given orally. The hazards of anticoagulant therapy in this condition were recognized.

Eleven patients have been treated with penicillin and dicumarol. The penicillin dosage ranged from 200,000 units daily given intramuscularly, usually at three-hour intervals, to 1,000,000 units given continuously intramuscularly or intravenously. Dicumarol was given in initial doses of 4 or 5 mg. per kilogram with subsequent daily doses of 1.5 mg. per kilogram as necessary to maintain the prothrombin time between 25 and 50 per cent of normal.

Seven patients recovered and have remained well for intervals of from seven to fourteen months. One patient died after a few days of therapy, possibly from cerebral hemorrhage. Two patients relapsed promptly after completion of therapy, but one of these left the hospital against our wishes before the minimum six-week course, which we have always advocated, was completed. A fourth patient was never brought under control, even with large doses of penicillin and intermittent dicumarol for more than six months. The first patient treated after being well for almost a year had a tooth with a periapical abscess extracted. Prior to the extraction the patient received 15,000 units of penicillin every three hours for five doses and after the operation, the same

amount at the same intervals for fifty-four hours. Within a week he again had positive blood cultures and fever.

Seven patients received penicillin alone in similar doses. One with inadequate therapy, a total of 800,000 units, died of cardiac decompensation two months later, and the vegetations were almost completely healed. Another has been well for a year. She received no dicumarol because of vaginal bleeding present at the time of admission. The other five patients did not respond to treatment, and four have died. The status of the fifth is unknown at present. In these cases, dicumarol was not given because negative blood cultures and control of fever were not attainable, and, after our early experience with the patient who died, we have withheld dicumarol until either a negative blood culture is obtained or the fever controlled. The differences in the results in the two groups are not as important as they seem; therefore, since after the first four patients, only those who were responsive to penicillin received dicumarol. Of the total eighteen patients, eight have recovered, and one of these has again acquired the disease. From our experience to date, we have acquired certain impressions and tentative conclusions.

1. Treatment with penicillin alone or where combined with dicumarol should be persisted for a minimum of six weeks.

2. This therapy has given decidedly better results than anything previously employed.

3. Despite our results favoring combined therapy, we remain decidedly skeptical that anticoagulant therapy offers any advantages, and it is unquestionable that anticoagulant therapy carries with it grave hazards in subacute bacterial endocarditis.

4. Penicillin therapy for fifty-four hours after removal of a focus of infection is *inadequate* to prevent the development of subacute bacterial endocarditis.

5. Seven of our patients who were not cured had received distinctly inadequate therapy before coming under observation, whereas only one of these eight who did recover had had any penicillin prior to ours. All but one of the inadequately treated patients had become subjectively and objectively improved under the original penicillin therapy. We raise the question as to the possible great significance of this point. Although a conclusion regarding it is not justified, it may be important, and an explanation is as yet wanting since in all but one of these patients, the organism was still sensitive *in vitro*.

DISCUSSION

DR. W. S. MIDDLETON, Madison, Wis.—The introduction of penicillin in the treatment of subacute bacterial endocarditis is a tremendous advance. I have lived through an unwholesome record of 126 consecutive deaths from subacute bacterial endocarditis. I am not boasting but am merely stating this as an experience of the past. In the experience of most workers in acute bacterial endocarditis. The route and dosage will be subject to further study. Just one small detail will be called to your attention. The continuous administration of penicillin, either intramuscularly or intravenously, presupposes the use of tubing. At the Medical

Research Council in Great Britain there was reported a reduction of the potency of penicillin passing through tubing other than Latex by about 50 per cent. In other words, if you are going to employ a continuous method of administration, do not use red or black rubber tubing, use latex.

DR. L. N. KATZ, Chicago, Ill.—It is interesting that the report of the Madison group is in agreement with the experience we have had at the Michael Reese Hospital, a preliminary report of which was made to this Society last year.

We have treated seventeen unselected successive patients with penicillin alone (without any combined anticoagulant). Of these seventeen, fourteen were "cured"—some have remained well and at work for over a year. Two patients died during therapy, one from a cerebral hemorrhage and the other from heart failure confirmed at necropsy. Of the other fifteen patients, only one was a treatment failure.

We gave as much as 158 million units of penicillin for as long as eight weeks. Ordinarily we gave from 200,000 to 400,000 units per day for from three to six weeks. We treated one patient with a resistant case with 2,500,000 million units daily toward the end of his course, and on the last three days we gave him para-amino hippuric acid to raise the blood level further. This patient has now recovered and is quite well and at work. One of the recovered patients died of heart failure eight months after therapy was finished. Necropsy confirmed the presence of a healing lesion.

The statistics in the literature and our own experience demonstrate that the addition of anticoagulants is without value. The rate of recovery is as high with penicillin alone as with penicillin and a anticoagulant, when penicillin is administered in large enough dosage for a long enough time, provided that the blood level is maintained high continuously. We used intermittent intramuscular injections given every hour, twenty-four hours a day. Penicillin is excreted very quickly and injections even two hours apart are not adequate to maintain a continuous high blood level. With the large doses we use continuous therapy. This must be maintained constantly day and night throughout the period of treatment.

I think the problem that remains in subacute bacterial endocarditis is the treatment of the penicillin-resistant organisms. In the course of time it may turn out that organisms will be found so resistant that penicillin may be ineffective.

DR. MILTON LANDOWNE, Bethesda, Md.—I would like to ask a question in relation to repeated courses of treatment with penicillin. In the first patient who had been well for almost a year, and in whom bacteremia developed a week after dental extraction despite penicillin, did the blood cultures show the same organism that had been present before? If so, did it have the same degree of in vitro sensitivity to penicillin as it did before? And bearing on this same point, have you any data as to whether the in vitro sensitivity of the organisms to penicillin was altered by the administration of penicillin in the seven patients who, having had previous "inadequate" therapy, failed to respond to your treatment?

DR. HAROLD FEHL, Cleveland, Ohio.—Could treatment be continued in the patient who was sensitive to penicillin?

DR. M. A. BLANKENHORN, Cincinnati, Ohio.—Has Doctor Meyer an opinion on the role of sulfa drugs in resistant penicillin cases?

DR. MEYER (closing).—In the patient who was cured and then relapsed following dental extraction, I cannot tell you whether the organism was identical with that of his first illness. On both occasions, however, the organism was penicillin sensitive and sensitive to 0.30 of a unit per cubic centimeter. It may have been sensitive to a greater dilution, but we did not go below that concentration in testing the sensitivity.

As to the second question, in none of our seven patients do I have information about penicillin sensitivity prior to the time patients came under our observation. Only one was treated by a group where they probably tested the organism for penicillin sensitivity. The second patient was given three million units but did not respond. We could never effect a sterilization of the blood stream.

Answering Doctor Feil's question regarding penicillin sensitivity, we have not observed patient sensitivity to penicillin that required our stopping therapy.

In only one patient was the combination of penicillin and a sulfonamide employed, and without benefit. The dose of penicillin in this case was to 1,000,000 units a day. It does not seem likely that the combination will be especially beneficial, but that is just an opinion.

By May of this year we were fairly convinced that if the patient would not respond to 200,000 units of penicillin a day he would not respond to larger doses. We have certainly changed our minds about that. You will find an occasional patient who will respond to much larger doses than who failed with small doses. We realize that this is not a large series of patients; there are only eighteen. Some of these impressions may not hold, but in 1944 we thought it was a simple matter to cure subacute bacterial endocarditis with penicillin. It is becoming increasingly difficult. We are not getting the same results with the same doses. Perhaps it is pure chance in a small series of cases. Time will tell.

A STUDY OF BERIBERI HEART DISEASE AT THE CINCINNATI GENERAL HOSPITAL

C. F. VILTER, M.D. (BY INVITATION), M. A. BLANKENHORN, M.D.,
R. S. AUSTIN, M.D. (BY INVITATION), AND I. M. SCHEINKER, M.D.
(BY INVITATION), CINCINNATI, OHIO

In five years at the Cincinnati General Hospital we have recognized beriberi heart disease in twelve patients with cardiac failure. The combined skills of the nutritionist, cardiologist, neurologist, and neuropathologist have been applied to the study of these patients. The following conclusions may be drawn.

Beriberi heart disease in the United States is likely to be missed if one follows strictly the description given by Wenckebach. Although the syndrome of a failing heart with signs of fast circulation occurs and gives a vivid picture, the more common type resembles any other type of degenerative heart disease. Therefore, other criteria have been established to aid in the recognition of beriberi heart disease. Of greatest importance are (1) the elimination of other etiologic agents, (2) a thiamine-deficient diet for longer than three months, (3) the presence of other evidence for nutritional failure, particularly neuritis or pellagra, (4) enlarged heart with sinus rhythm, (5) dependent edema, (6) elevated venous pressure, (7) minor electrocardiographic changes, and (8) recovery with decrease in heart size or necropsy findings consistent with beriberi but not with other types of heart disease.

The onset was sudden in some cases, gradual in others, and healing occurred in the same fashion. The response to thiamine was not dramatic except in one patient. The duration and severity of deficiency probably played a part in the reversibility or irreversibility of the process.

In some instances the condition of the patient was critical at the time of admission. Five of the patients died and necropsy was performed in three of these. In addition to lesions previously reported for beriberi, degenerative changes were found in the central, peripheral, and autonomic nervous systems. The outstanding lesions were (1) degenerative alterations in the cells of the sympathetic nuclei and fibers in the lateral horns of the upper cervical segments of the spinal cord, (2) damage of the spinal cord roots, (3) far-advanced degeneration of the vagus nerves and of some of the peripheral nerve trunks.

These lesions support the theory that the syndrome of beriberi heart disease is associated with neuritis, involving the central and peripheral autonomic nervous systems.

DISCUSSION

DR. EMMET F. PEARSON, Springfield, Ill.—I should like to ask whether blood protein determinations were made on these patients?

DR. GORDON B. MYERS, Detroit, Mich.—In some of our cases of beriberi we have found a sharp inversion of the T wave in Leads V_1 to V_3 which disappeared after thiamine therapy. Have you utilized multiple precordial leads in your cases?

DR. G. E. WAKERLIN, Chicago, Ill.—Were blood pyruvic acid determinations made on these patients? Also was vitamin B complex therapy employed in addition to thiamine?

DR. VILTER (closing).—In each instance there were some blood protein studies; sometimes we measured only the total protein. In practically every instance the serum protein was low. One could not say, however, there was a relative lowering of serum albumin. The total plasma protein was always under 6 and in most instances it was between 4.5 and 5. The dilution factor seemed more important than protein deficiency.

In a few patients we made serial electrocardiographic studies. The electrocardiograms tended to revert to normal as the patient recovered, if he did recover. With progression of the disease there was no progression in the severity of the changes in the electrocardiogram.

We did not make blood pyruvic acid studies in these patients. These studies extended over five years, and we did not use the pyruvic acid level as a routine clinical laboratory test.

We did not use other members of the B complex group in treatment of the usual case. I think that is very important in control studies. In those cases in which it is feasible we like to have first a control period of observation with the patient at rest in bed in order to tell just what bed rest will do, since many previous investigators have simply reported that patients were put to bed and given thiamine. Thiamine itself, as I said before, did not give dramatic results. Bed rest alone may produce moderate improvement. After a suitable period of rest, we gave thiamine alone unless the patient was in a critical stage of nutritive failure. If so, we used ascorbic acid, niacin, and other members of the B complex group. It is wise also to give some natural source of B complex such as brewer's yeast or crude liver extract to these patients since mixed deficiency is so much the rule as to serve as one of the criteria for the diagnosis. Above all, the dietary should be restored and augmented so as to yield from 3,000 to 4,000 calories, 120+ Gm. of protein, and adequate vitamins and minerals.

FOLLOW-UP STUDIES OF PATIENTS WITH CARDIAC WOUNDS

PAUL H. NOTH, M.D., DETROIT, MICH.

This group of patients with penetrating cardiac wounds was restudied for the particular purpose of obtaining electrocardiograms at a late enough period so that the masking effect of pericarditis should have subsided leaving patterns which could be presumed to be due to the wounds themselves. The electrocardiographic findings were compared with other clinical features.

Eight patients were completely examined by me at periods varying between five and thirty-six months and averaging nineteen and four-tenths months following the wounds. Five complained of a variety of indefinite symptoms and three had no symptoms. However, the latter all had abnormal electrocardiograms, whereas three of the five patients with symptoms had normal tracings.

Abnormal physical findings were absent in all except two patients. Venous pressure was normal in the seven patients tested for this.

Ten patients had roentgenologic studies. Roentgenograms were entirely normal in eight. Three of these eight patients had abnormal electrocardiograms. Two patients exhibited abnormal quality of cardiac pulsation as observed fluoroscopically. Both had abnormal tracings.

Twelve patients were followed electrocardiographically for periods averaging eighteen months. All had precordial leads and nine had six precordial leads (Wilson type of central electrode) and augmented unipolar extremity leads. This group is a notable addition to the twenty-three cases of penetrating heart wounds with published electrocardiograms recorded in the literature, without ligation of a coronary artery, which were followed for three months or longer. The average follow-up period was only five and six-tenths months.

In the present study four of the six patients with left ventricular wounds had abnormal electrocardiograms. All showed abnormalities of the T waves in Leads I and V_6 , and sometimes also in Leads II, V_4 , and V_5 . The electrocardiogram of one of the three patients with right ventricular wounds was abnormal and showed incomplete right bundle branch block, right ventricular premature systoles, and T wave abnormalities most marked in Leads V_3 to V_5 . The electrocardiogram of a patient whose wound was of the pulmonary vein and pericardium showed changes characteristic of acute pericarditis, apparently due to persisting empyema and complicating pericarditis. Two patients with less severe wounds not requiring operation had normal tracings.

In general, the electrocardiograms exhibit the most persistent objective abnormalities; consequently, they are of value as an indication of the need for some caution in the management of such cases. When the electrocardiogram was normal, other objective studies also were normal and the persisting symptoms strongly suggested a complicating cardiac neurosis. These late electrocardiograms in this series and in the literature confirm the impression that heart wounds in themselves often produce localizing electrocardiographic changes and that these changes are usually seen in the leads reflecting also the common types of involvement of the right or left ventricles. Multiple precordial leads may add useful localizing information.

THE PRECORDIAL ELECTROCARDIOGRAM IN HIGH LATERAL MYOCARDIAL INFARCTION

FRANCIS F. ROSENBAUM, M.D., FRANK N. WILSON, M.D., AND
FRANKLIN D. JOHNSTON, M.D., ANN ARBOR, MICH.

Observations have been made in eight patients whose routine standard and precordial electrocardiograms showed only slight or suggestive evidence of myocardial infarction, whereas more extensive exploration of the upper left anterior, lateral and posterolateral aspect of the thorax disclosed more diagnostic changes. Four of the seven patients gave a history of acute coronary thrombosis a few days to one year prior to the observations. Two had moderately severe congestive heart failure and two had severe peripheral vascular disease; in these four patients the time of occurrence of the infarction was less certain.

In five patients the most characteristic changes of myocardial infarction were recorded in the vertical line of V_3 and V_4 but from one to three interspaces higher than the usual level; these we have designated as high anterolateral infarction. In two patients the most marked changes occurred in leads from the anterior or mid-axillary lines but two or three interspaces higher than the usual V_5 or V_6 ; these we have termed high lateral infarction. In one case the most striking changes were observed in records taken high in the posterior axillary and left scapular lines and were attributed to a high posterolateral infarct. The observations made were not the same in all patients since only as the study progressed were the most advantageous points for exploration revealed.

Some patients with high anterolateral or posterolateral infarction displayed either small Q waves, slightly inverted T waves or both, but others displayed only minor changes in the standard and unipolar left arm (V_L) leads. These leads showed more significant changes in those patients with high lateral infarction, suggesting that the involved area faced the left shoulder more directly. In the entire group of patients the routine precordial leads (V_1 through V_6) either disclosed some diminution in the size of the R waves or failed to show the usual rapid increase in size of the R waves as the exploring electrode was moved from right to left, but prominent Q or QS waves did not appear.

It is suggested that observations of the kind reported here be made, not routinely, but in those instances in which the history, limb leads, or both are suggestive of myocardial infarction and in which the usual precordial leads fail to yield conclusive evidence of this condition.

DISCUSSION

DR. GORDON B. MYERS, Detroit, Mich.—About a year ago we encountered two patients who had diagnostic signs of infarction in Goldberger Lead aV_L with inconclusive findings in other leads. Both went to autopsy and a diagnosis of high lateral myocardial infarction was confirmed. Since that time we have made use of high axillary leads comparable to Doctor Rosenbaum's, but no opportunity for further autopsy confirmation has been offered.

In the interpretation of Goldberger Lead aV_L , one must bear in mind that in vertically placed hearts the mitral orifice may face toward the left arm, resulting in the transmission of the potentials of the left ventricular cavity to the left arm, causing a Q wave and inverted T wave in Lead aV_L . This finding is more common when the electrocardiogram is taken in the recumbent posture and tends to disappear when the electrocardiogram is taken in the sitting position.

ELECTROCARDIOGRAPHIC DIAGNOSIS OF RIGHT VENTRICULAR LESIONS WITH ESPECIAL REFERENCE TO THE FINDINGS IN THE WILSON PRECORDIAL AND GOLDBERGER EXTREMITY LEADS

GORDON B. MYERS, M.D., AND BERT E. STOFER, M.D. (BY INVITATION)
DETROIT, MICH.

This study is based upon twenty cases in which an electrocardiographic diagnosis of right ventricular hypertrophy was confirmed at autopsy and upon forty-one additional cases in which an electrocardiographic diagnosis of "acute

right ventricular strain" was supported by the post-mortem finding of right ventricular dilatation which was associated with extensive pulmonary infarction, consolidation, or atelectasis in twenty-two of the cases and was secondary to acute left ventricular failure in nineteen. The standard leads, together with $V_{2, 4, 6}$ were taken on all cases; in addition, leads $V_{1, 3, 5}$ and the Goldberger extremity potentials were taken on thirty-two cases. Post-mortem examination included injection of coronary circulation in thirty-two cases; determination of the ratios of the right and left ventricular muscle mass in thirty-eight cases. This series was augmented by a larger group of cases in which the diagnosis rested on clinical grounds.

The electrocardiographic pattern of right ventricular hypertrophy is characterized by a prominent R wave with late intrinsicoid deflection in leads V_1 , V_2 and aV_R ; a relatively small S wave in $V_{1, 2}$ and relatively large S wave in $V_{5, 6}$; and depression of the RS-T junction with diphasic to inverted T waves in Leads V_1 , V_2 , and usually V_3 . The findings were essentially the same irrespective of whether the right ventricular hypertrophy was secondary to congenital heart disease, mitral stenosis, or chronic pulmonary disease. The electrocardiographic pattern of "acute right ventricular strain" was characterized chiefly by the abrupt development of sharply inverted T waves with convex RS-T segments in $V_{1, 2, 3}$ followed by rapid changes in serial curves. "Right ventricular strain" was differentiated from anteroseptal infarction by the absence of Q wave pattern and by the fact that the T wave changes were less marked in V_3 than in V_2 and V_1 . Right bundle branch block, which may also occur in association with right ventricular hypertrophy or "acute right ventricular strain," was not included in this study.

DISCUSSION

DR. L. N. KATZ, Chicago, Ill.—I think Doctor Myers is to be congratulated upon an analysis of a very difficult field. All of us who have had experience in electrocardiography have come to realize that we cannot readily diagnose right heart strain from the ordinary limb leads. Unless we know clinically that the right heart is enlarged it is often impossible to make the diagnosis from the electrocardiogram alone. Such a diagnosis of right heart strain can be made from the limb leads if S_1 and S_2 are very deep. The diagnosis can often be made from the appearance of the chest leads CF_1 , CF_2 , and CF_3 , especially when the R/S ratio decreases from CF_3 to CF_1 . I am still reluctant to believe that the so-called unipolar leads, which are really not unipolar, can be helpful.

DR. FRANCIS F. ROSENBAUM, Ann Arbor, Mich.—I feel that there are some situations in which the findings reported by Doctor Myers and Doctor Stofer may be confusing, particularly in the differentiation of right ventricular hypertrophy and right bundle branch block. This is especially the case when we are dealing with incomplete right bundle branch block. We have not found that the unipolar lead from the right arm (V_R) is of particular help since large R waves are seen in this lead in both conditions. It is true that this R wave is more sharp and peaked in right ventricular hypertrophy, whereas in right bundle branch block it is more likely to be notched, broad, or slurred.

DR. MYERS (closing).—We have had the same difficulty as Doctor Rosenbaum in distinguishing between the classical pattern of right ventricular hypertrophy and right incomplete bundle branch block. However, our cases of incomplete right bundle branch block who have come to autopsy showed right ventricular hypertrophy, so the distinction is mainly of academic interest.

IRREVERSIBLE AND SLOWLY REVERSIBLE CHANGES IN SEVERE
MALNUTRITION

EMMET F. PEARSON, M.D., SPRINGFIELD, ILL.

Of the 567 white civilian patients admitted with the primary diagnosis of malnutrition to the United States Army hospitals in Manila after its liberation, many died in spite of efforts to reverse the degenerative processes. Other patients slowly recovered from various physiologic disturbances and in some there was certain evidence of permanent tissue damage.

Among the degenerative processes observed were nerve changes; changes in the retinae, optic nerve, and lens, causing failing vision; evidence of myocardial damage; skeletal muscle weakness; and certain evidence of damage to the viscera.

DISCUSSION

DR. C. F. VILTER, Cincinnati, Ohio.—Doctor Blankenhorn asked me to discuss this paper for him because he had to leave.

I would like to reply to the questioning of thiamine deficiency as the cause of beriberi heart disease in our patients. Although the plasma proteins of every patient were low, it seems obvious that this was due to the dilution factor of cardiac edema rather than to primary protein deficiency. Although most of these patients were on a low protein, thiamine-deficient diet, the plasma proteins were restored to normal coincident with thiamine administration and cardiac compensation, a much more rapid restoration than could be afforded by dietary protein supplements. The single patient, who showed a dramatic cure following thiamine, exemplifies the fact that protein deficiency is not causal in the disorder. This patient had massive edema and the total proteins were 6.4 Gm. per cent. After three days on the control diet, she was given thiamine, and diuresis began in twenty-four hours. In a week she lost twenty-one pounds of edema fluid. Protein intake was low during this period. Studies made in the past by Wenckebach, Weiss, Wilkins, Keefer, and many others have definitely indicated that thiamine deficiency is the cause of, and administration of thiamine the cure for, beriberi heart disease.

I would like to ask if Doctor Pearson has made any studies which determine that protein alone causes remission of beriberi.

DR. PEARSON (closing).—All of the cases I saw that were called "wet" beriberi had hypoproteinemia. That is the only evidence I have to go on. I think Doctor Vilter stated this morning, that his patients with beriberi heart had hypoproteinemia, from 4 to 6 Gm. I believe a finding of 6 Gm. is definite hypoproteinemia. One gram deficiency in the blood represents considerable deficiency in the body in general. I have not made any further studies to determine whether hypoproteinemia with edema and beriberi are the same thing, but if all of a group of patients with beriberi have demonstrable hypoproteinemia, I consider that sufficient evidence. The confusing thing is that most individuals who are forced to remain on a low thiamine diet are also on a low protein diet. These people at Santo Tomas had nothing but a carbohydrate diet. One of the things that may happen when there is a prolonged carbohydrate diet without protein is a loss of the mechanism for digestion of protein. The trypsin is measurably decreased. If the mechanism for protein digestion is not worked regularly, it will cease to be efficient.

THE TOTAL SOLIDS, FAT, AND NITROGEN IN THE FECES OF
PERSONS WHO HAVE UNDERGONE PARTIAL GASTRECTOMY WITH
ANASTOMOSIS OF THE ENTIRE CUT END OF THE STOMACH
TO THE JEJUNUM (POLYA ANASTOMOSIS)

ERIC E. WOLLAEGER, M.D. (BY INVITATION), MANDRED W. COMFORT, M.D.,
JAMES F. WEIR, M.D., AND ARNOLD E. OSTERBERG, PH.D.
(BY INVITATION), ROCHESTER, MINN.

A small percentage of patients subjected to partial gastrectomy for lesions of the stomach and duodenum experience difficulty in gaining weight after operation. The inability to gain weight appears to be due in part to the reluctance of the patient, because of his distress, to take an adequate diet. However, it seemed possible that inability to gain weight also might be due in part to impaired utilization of ingested food. This possibility led to the following metabolic studies.

A series of thirteen persons who had not undergone gastric surgery were fed a diet containing 208 Gm. of fat, 97.5 Gm. of protein, 140.5 Gm. of carbohydrate and 2,823 calories per day. A second series consisting of ten persons who had undergone partial gastrectomy with anastomosis of the entire cut end of the stomach to the jejunum were fed the same diet. A third series of four persons who had undergone partial gastrectomy with anastomosis of the entire cut end of the stomach to the jejunum also were studied; these, however, were fed weighed diets of lower fat content. These diets contained from 100 to 125 Gm. of fat and from 90 to 100 Gm. of protein. The earmine marker technique was used to begin and to end the period of study. Seventy-two-hour samples of stools were collected from the patients in Groups 1 and 2; longer periods of collection were used in Group 3. The total solids, fat, and nitrogen were measured in all samples.

Analysis of the data obtained disclosed the following:

1. Nearly all persons studied who had undergone partial gastric resection with anastomosis of the entire cut end of the stomach to the jejunum lost more fat in the stool when taking either a diet containing a moderate or a high amount of fat than did those who had not undergone any operation on the stomach. This loss occurred in patients who were not having symptoms referable to the digestive tract but tended to be greater among patients who had postoperative digestive complaints.

2. Some patients with partial gastrectomy and the anastomosis of the type described lost more nitrogen in the stool than did those who had not had an operation on the stomach.

3. In most instances the amount of fat and nitrogen in the feces in excess of normal was not large, but this higher excretion may be one of the causes of inability to gain weight, especially when the intake of food is limited by the patient's postoperative distress.

DISCUSSION

DR. ELMER L. SEVRINGHAUS, Madison, Wis.—I would like to ask if in any of these patients there are any data indicating the value of special types of fat, such as milk fat compared with the other fats in the diet?

DR. G. E. WAKERLIN, Chicago, Ill.—Was the effect of pancreatic enzymic preparations studied in these partially gastrectomized patients?

DR. ROBERT M. STECHER, Cleveland, Ohio.—How many days postoperative were these studies made? Was there a variation with the increase in the time interval after operation?

DR. WOLLAEGER (closing).—In answer to the question concerning the utilization of different kinds of fat, we have tried only one type of diet in which the fat consisted of cream, butter, and meat fat. We have not studied the utilization of other kinds of fat.

We have not done enough work with pancreatic preparations to make any statement about their effect in regard to the utilization of fat by persons who have undergone a gastrectomy. We studied one patient who had a rather large fat loss in the stool and an achlorhydria following the Ewald test meal. He was given large doses of powdered pancreatin during one three-day period of study and the fat loss in the stool was reduced to normal amounts. We have studied no other patient in this manner.

The length of time following operation that our patients with gastrectomy were studied varied from three months to eight years.

THE EFFECT OF ALUMINA GEL UPON THE ABSORPTION OF
NUTRIENT SUBSTANCES FROM THE INTESTINAL TRACT

WILLIAM S. HOFFMAN, M.D., AND HATTIE A. DYNIEWICZ, Ph.C.

(BY INVITATION), CHICAGO, ILL.

To study the possible effect upon intestinal absorption of therapeutic doses of alumina gel, tolerance curves were determined in hospital control subjects of vitamin A, ascorbic acid, glucose, fats, and amino acid, first under control conditions and then after the ingestion of alumina gel. In seventeen subjects, the vitamin A tolerance curves, obtained after ingestion of 75,000 I.U., were slightly but significantly depressed if 1 ounce of alumina gel (Amphojel) was given simultaneously. This depression did not occur if alumina gel was given every two hours for one or more weeks before the second tolerance curve was obtained. The depressed absorption (if that was the cause of the flattened curve) was therefore not due to any tanning action of the alumina gel. With aluminum phosphate gel no depression of the vitamin A tolerance curves was seen. That the slight depression of vitamin A tolerance curves has no nutritional significance is indicated by the finding that in thirty-three patients with peptic ulcer who had been on alumina gel management for two or more weeks, the plasma vitamin A and carotenoid concentrations showed no deviation from the normal.

Tolerance curve comparisons were made in twelve subjects for amino acids, in twelve subjects for ascorbic acid, in fifteen subjects for glucose, and in ten subjects for neutral fat. In the first three, statistically insignificant flattening of the tolerance curves occurred; in the case of fat, there was no demonstrable effect of alumina gel. It is possible that alumina gel, by reducing the amount of available phosphate, may interfere slightly with phosphorylation where that process is involved in intestinal absorption.

BOOK REVIEWS

Familial Nonreaginic Food-Allergy. By *Arthur F. Coca*, M.D., Medical Director, Lederle Laboratories. Charles C Thomas, Springfield, Ill., 1945. Price \$3.75. Cloth with 153 pages.

Year Book of Industrial and Orthopedic Surgery. Edited by *Charles F. Painter*, M.D., Orthopedic Surgeon to the Massachusetts Women's Hospital and Beth Israel Hospital, Boston, Mass. The Year Book Publishers, Inc., Chicago, Ill., 1946. Price \$3.00. Cloth with 432 pages.

Announcement

The Blood Transfusion Association of New York City will resume grants-in-aid for research in the field of blood and blood substitutes in relation to transfusions.

Those interested in obtaining such grants should write, giving full information concerning their projects, to the Chairman of the Research Grants Committee, 2 West 106th Street, New York 25, N. Y.

DEWITT STETTEN, M.D., PRESIDENT

REFLEX SHORTENING OF THE ESOPHAGUS IN THE EXPERIMENTAL ANIMAL WITH THE PRODUCTION OF ESOPHAGEAL HIATUS HERNIA

F. L. DEY, M.D., N. C. GILBERT, M.D., RUTH TRUMP, B.S., AND
R. C. ROSKELLEY, M.D.
CHICAGO, ILL.

THERE are many reasons for the conclusion that esophageal hiatus hernia is an acquired condition, occurring most frequently in the fourth decade of life and later when the tissues about the hiatal orifice have become more relaxed. In a large part of the cases, an increase in intra-abdominal pressure may be assumed to be the determining cause.

But cases in which the hernia cannot be ascribed to an increase in intra-abdominal pressure do occur. These follow a pattern suggesting that some neurogenic factor is valent. Von Bergmann and Goldner,¹ and, later, Hurst² suggested that in many cases the occurrence of hiatus hernia might be due to traction of the stomach upward, due to a shortening of the esophagus in response to some vagal stimulation. Von Bergmann and Goldner quoted experiments by Kuckuck, showing that electrical stimulation of the vagus trunk caused a longitudinal contraction of the esophagus in the rabbit, which was capable of pulling the stomach up through the hiatal opening in the diaphragm.

This work was repeated in experiments upon the dog,³ using Michel clips fastened to the stomach wall and the lower part of the esophagus to indicate their position in the roentgen plate. After control plates were made, the vagus trunk was stimulated electrically and pictures were taken during the stimulation. The esophagus was shown to be shortened by the stimulation of the vagus, and the stomach was pulled up to the diaphragm. These experiments were performed with the chest cavity closed, and the stomach was not pulled up through the hiatal orifice. Later, with the chest cavity opened, the cardiac end of the stomach was pulled up through the hiatal ring.

The present work was undertaken in order to determine whether or not vago-vagal reflexes could be elicited from mechanical stimulation of the peritoneum and viscera of the upper abdomen, which would reflexly shorten the esophagus and produce an esophageal hiatus hernia.

Dogs weighing between 7 and 10 kilograms were utilized in this study. The dogs were either decerebrated or else anesthetized by the method of Grehant,

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which, in our experience, is less prone to inhibit viscero-visceral reflexes than are some other anesthetics. A mechanical respirator was inserted in the trachea, and the vagi were isolated. With the dog on its right side, the left side of the chest was opened by resecting the ninth and tenth ribs, and a recording lever was attached to the esophagus, as shown in Fig. 1. A standard kymograph was utilized for recording deviations of the lever caused by shortening or lengthening of the esophagus. At the beginning of each experiment and at intervals throughout the experiment, control readings were made by stimulating the left vagus nerve in the neck by means of a Harvard inductorium.

A reflex shortening of the esophagus by means of various stimuli was demonstrated almost invariably from stimulation of one source or another in the upper abdomen. The ease with which these reflexes occurred varied inversely with the degree of depression caused by the decerebration or the anesthesia and also with the absence of shock due to insufficient anesthesia.

Mechanical stimulation of the peritoneum by scratching with a pointed instrument caused a reflex shortening of the esophagus in many of the animals. The effect of such stimulation is shown in Fig. 2. In this figure, as elsewhere, contraction or pulling up of the esophagus is shown by the upstroke, and relaxation or lengthening of the esophagus is shown by the downstroke. This reflex from irritation of the peritoneum was obtained in only some of the animals, but, in those in which it did occur, it was readily reproducible.

Reflex contraction of the esophagus was most readily obtained by any manipulation of the liver. The mere insertion of a finger between the leaves of the liver, such as in locating the gall bladder, was usually a sufficient stimulus. That the reflex was not due to peritoneal irritation is indicated by its occurrence in animals in which a reflex was not observed by mechanical stimulation of the peritoneum elsewhere.

Traction upon the right lobe of the liver also caused a reflex contraction of the esophagus independent of peritoneal irritation (Fig. 3). In applying traction to the liver, the diaphragm was pulled down, and with it, the esophagus. The initial downstroke in Fig. 3 is due to the traction of the esophagus downward by the initial traction upon the liver, and the upstroke is caused by the resulting reflex contraction of the esophagus. If very gentle traction upon the liver was made, so as not to disturb the position of the diaphragm, a similar shortening of the esophagus occurred, but with no downstroke (Fig. 4).

A reflex shortening of the esophagus also occurred if the muscle fibers of a small area of the stomach were stretched manually (Fig. 5). The demonstration of a reflex resulting from the manual stretching of a small area of stomach wall obviated the introduction of variables resulting from the distention of the entire stomach.

Reflex shortening of the esophagus also occurred as a result of traction made upon the gall bladder or of overdistention of the gall bladder with normal saline solution, or from distention of the cystic duct by passing a fine artery snap into the cystic duct and spreading it (Fig. 6).

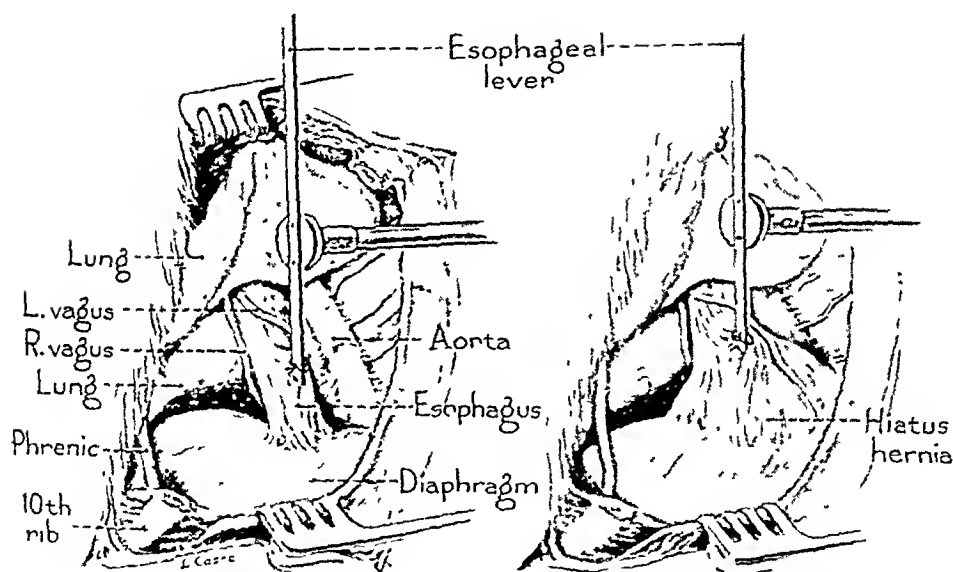


Fig 1.—Drawings showing arrangement of apparatus as set up to record shortening or lengthening of the esophagus. The right-hand figure shows the effect of shortening the esophagus. The cardiac end of the stomach is pulled up to the narial orifice.

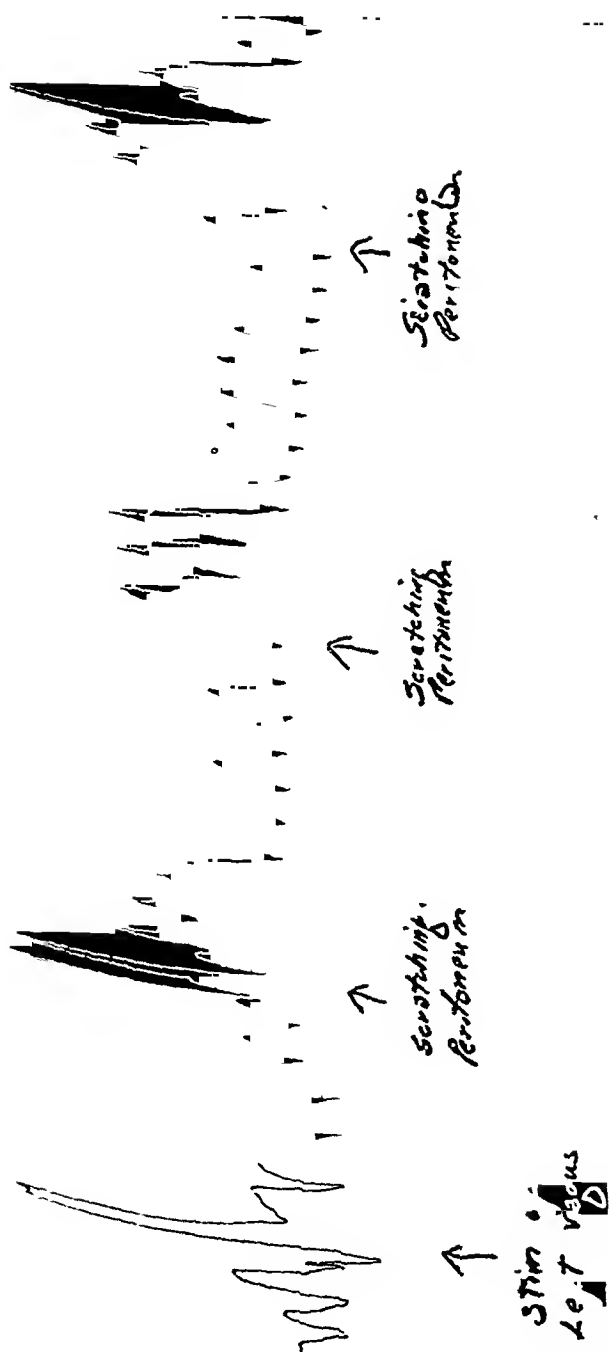


Fig. 2.—Upstroke shows shortening of the vagus due to electrical stimulation, followed by several incidents of reflex shortening of the esophagus after scratching the peritoneum with pointed instrument.

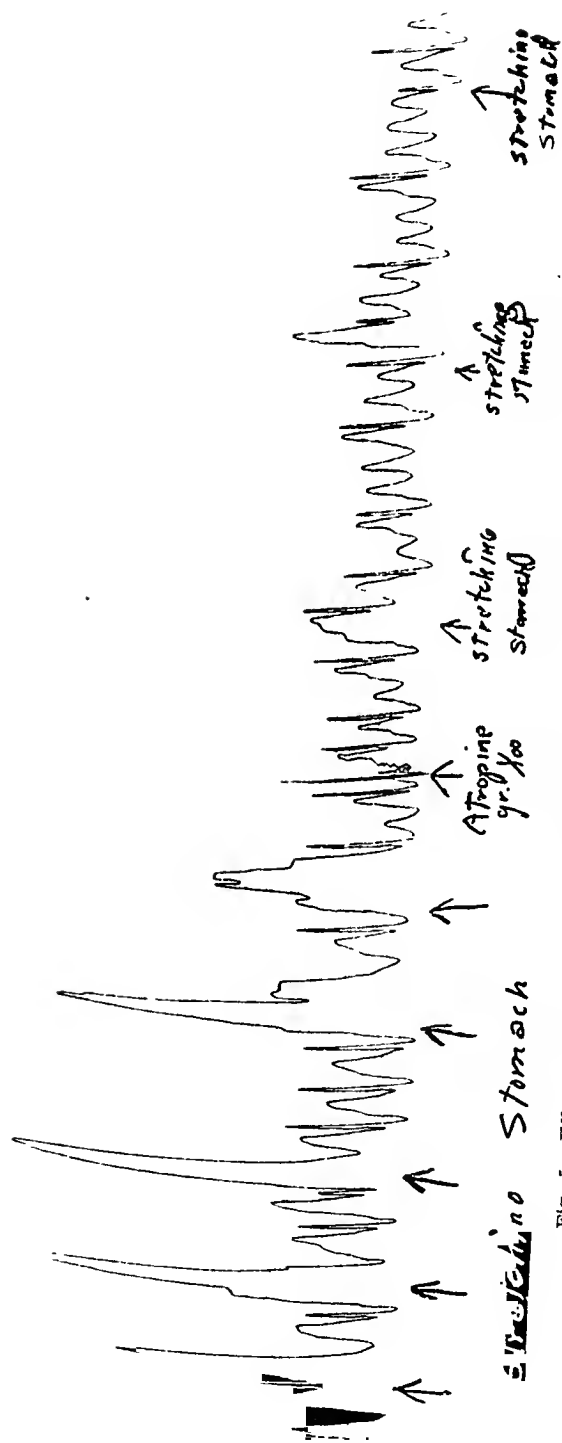


Fig. 5.—Effect of manually stretching small areas of the stomach wall. Reflex is abolished by atropine.

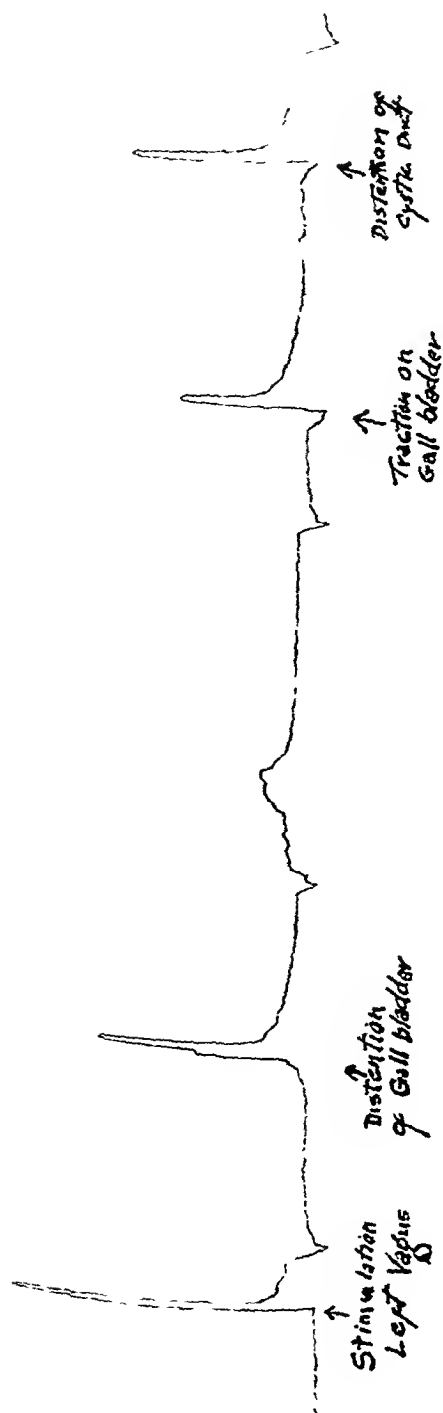


Fig. 6.—Reflex shortening of the esophagus due to distention of the gall bladder with normal saline, traction upon the gall bladder, and distention of the cystic duct.

CONCLUSION

Shortening of the esophagus with the production of a hiatus hernia due to traction upon the stomach in the dog results from electrical stimulation of the vagus nerve or reflexly from stimuli having their origin in the viscera of the upper abdominal cavity.

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A COMPARISON BETWEEN THE VALUES FOR PLASMA OR SERUM PROTEIN AS OBTAINED BY THE SPECIFIC GRAVITY AND THE MICRO-KJELDAHL METHODS

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MANY attempts have been made to perfect some simple and rapid method for the estimation of serum protein. The determination of nitrogen by the Kjeldahl procedure offers the most accurate means, but since it is time consuming and dependent on special equipment and requires a certain amount of skill, it has not proved adaptable in many instances where protein determinations would have been of great value in the physiologic assessment of subjects. In 1926, Barbour and Hamilton¹ described an accurate method for determining the specific gravity of serum or plasma. Moore and Van Slyke,² in 1930, found a high degree of correlation between the specific gravity of plasma and its protein content, and they expressed the relation between them by the formula:

$$\text{Total protein} = 343 (\text{specific gravity} - 1.007)$$

Experience has shown that certain precautions are necessary for accuracy in determining specific gravity. In modifications of Barbour and Hamilton's method in which mixtures of xylene and bromobenzene are used as the standard medium, care must be taken to prevent evaporation of these volatile substances. In addition, the density of the standard solution of potassium sulfate or copper sulfate must be carefully checked. In the method described by Kagan,^{3,4} accurate results can be obtained if sufficient attention is given to maintaining drops of serum of uniform size and if the temperature is carefully controlled, but accuracy may be reduced if these factors are ignored.

Recently, Phillips and associates⁵ described the use of copper sulfate solutions of different known densities in which a drop of serum or plasma either rises or falls, depending on its specific gravity. This method seemed to be particularly well adapted for use in field laboratories or on shipboard since no precision instruments are required. Uniformity in size of the drop is apparently unimportant, and no temperature correction is necessary because the coefficient of expansion of copper sulfate solutions approximates that of blood and plasma. Here again it is essential to use the utmost care in the preparation of the copper sulfate solutions of known specific gravity.

When plasma is used, it is necessary to apply a correction factor for the anticoagulant in the calculation of protein from the specific gravity. Experience in this laboratory has led us to the belief that many plasma specific gravity results are false because of inaccurate measurements of the volume of blood when taken in a syringe. For this reason it is preferable to use serum instead of

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plasma in order to eliminate slightly inconstant antieoagulant concentrations which make it impossible to apply an accurate correction figure.

Provided that all precautions are taken to ensure precise specific gravity determinations, the question next arises as to whether or not there is always a close correlation between the specific gravity of serum and its protein content. In 1942, Looney⁶ studied the relation between the specific gravity of serum and its protein concentration in a group of patients with schizophrenia and also in a group of normal subjects. He found that the correlation between the two was too low to permit the use of specific gravity determinations for accurate serum protein estimations. Similar observations were made by Zozaya⁷,⁸ and by Moon and associates.⁹ On the other hand, Atehley and co-workers¹⁰ have stated that in their experience the copper sulfate method of Phillips and associates⁵ for determining the specific gravity of plasma proved to be a simple and accurate method for the estimation of plasma proteins.

Several hundred protein determinations have been made in this laboratory since August, 1943, both by the micro-Kjeldahl method and by the copper sulfate specific gravity method of Phillips and associates.⁵ One hundred twenty-eight of these comparisons have been selected for presentation here, because it was felt that the technique used in this group was the most free from criticism. Serum was used in all of these cases to eliminate any possible changes in specific gravity which might be caused by variations in oxalate concentration. The micro-Kjeldahl determinations were done by digestion with a mixture of concentrated sulfuric acid, copper sulfate, and potassium sulfate, with the addition of potassium persulfate as an oxidizing agent. In ninety-five of the reported cases, distillation was done with a simple condenser, and the distillates were nesslerized and read in a Klett-Summerson photoelectric colorimeter. Thirty-three of the determinations were distilled with a Keys closed ammonia still, and the distillates were titrated using a Rehbürg burette. Since the results of the two methods showed no significant differences, they are considered together. Nonprotein nitrogen determinations were made by the Folin-Wu method.¹¹

For the specific gravity determinations by the method of Phillips and associates, the copper sulfate solutions used were prepared according to their directions, and the final standards were checked for accuracy by pycnometric determinations for the density of the actual solutions used. The serum total protein was calculated from the density by the formula:

$$\text{Total protein} = 343 (\text{specific gravity} - 1.007)$$

An analysis of these 128 comparisons is shown in Figs. 1 and 2. In Fig. 1, the differences found between the total proteins by the two methods are divided into groups covering 0.2 Gm. of protein per 100 c.c. of serum. The cases in which closest agreement between the two methods was found are shown in the center of the chart, with those in which the copper sulfate method gave lower results at the left of the center, and those in which the figures obtained from the copper sulfate method were higher at the right. It can be seen that in only forty-five instances, or 35 per cent, the variations between the results obtained by the two methods did not exceed ± 0.2 Gm. of protein per 100 c.c. of serum.

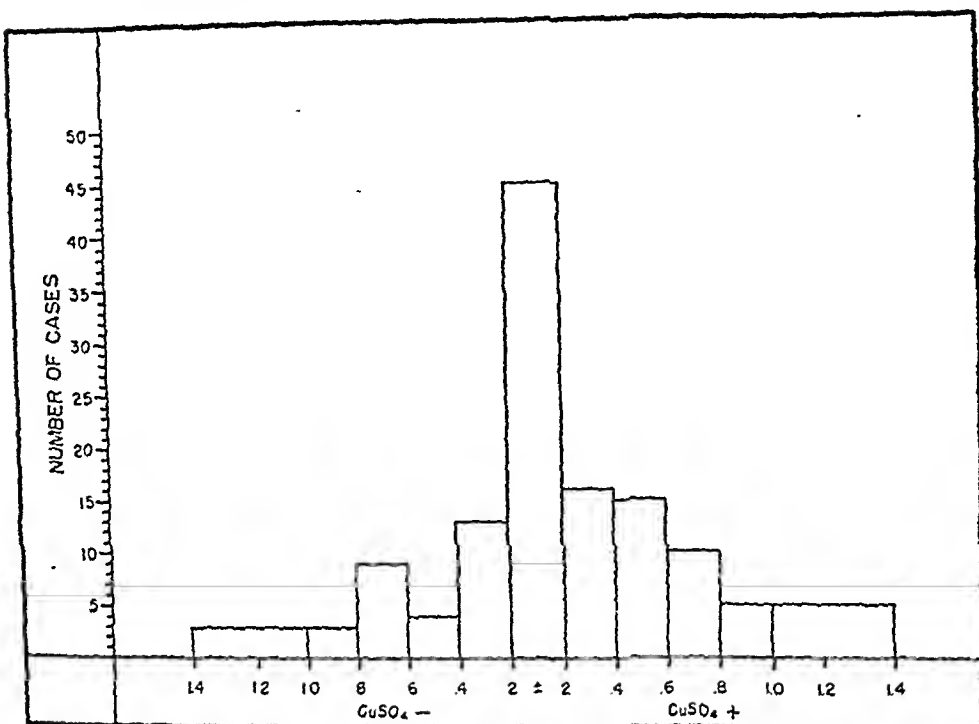


Fig. 1.—Differences between total proteins by micro-Kjeldahl and copper sulfate specific gravity methods.

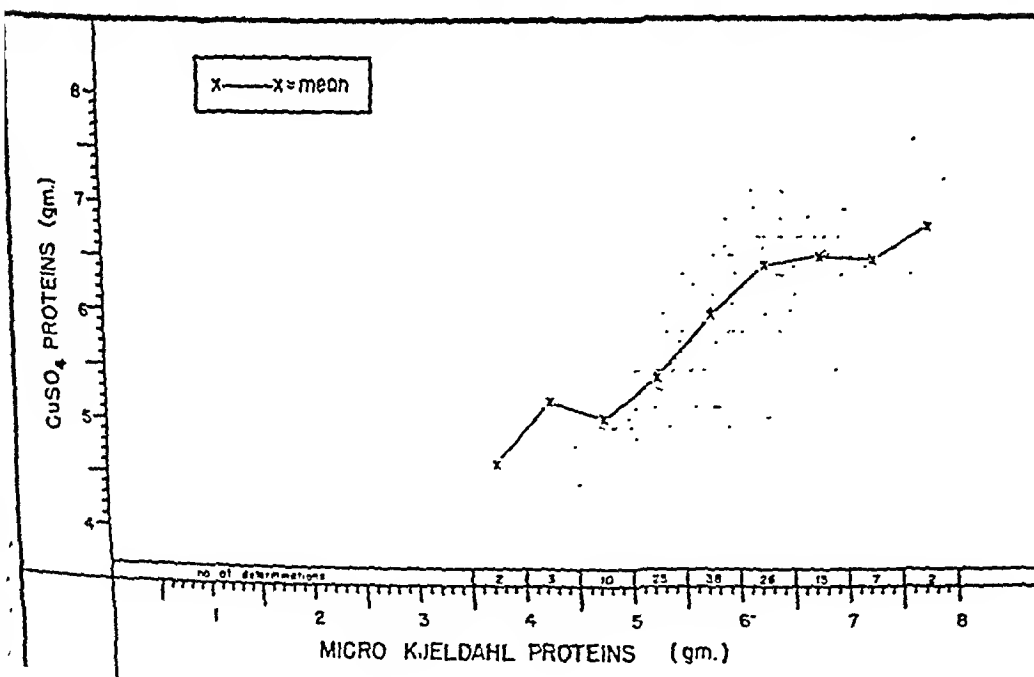


Fig. 2.—Correlation of the serum total proteins by copper sulfate specific gravity method with those by the micro-Kjeldahl method.

Duplicate micro-Kjeldahl results may be regarded as checks when the variations fall within this range, so that the two methods can be said to agree in forty-five of the 128 cases. In Fig. 1 are shown variations up to ± 1.4 Gm. of protein per 100 c.c. of serum, with the larger discrepancies occurring in decreasing numbers of instances.

In Fig. 2 is demonstrated the graphical correlation between the serum total protein as determined by the micro-Kjeldahl method and the value obtained by calculation from the specific gravity of serum. It can be seen that the results by the copper sulfate method are scattered over a fairly wide range when compared with the micro-Kjeldahl determinations on the same samples of serum. To plot the line $x-x$ in Fig. 2, the micro-Kjeldahl results were divided into groups covering 0.5 Gm. of protein per 100 c.c. of serum, and the mean copper sulfate value for the sera falling within each one of these micro-Kjeldahl groups was calculated. It can be seen that between protein values of 4.75 and 6.25 Gm. per 100 c.c. of serum the line $x-x$ follows a straight line very closely and that below and above these points there appears to be less correlation. However, the greatest differences from the mean occur in this group of patients and a better evaluation of correlation may be obtained by calculating the coefficient of correlation by the method described by Mainland.^{12, pp. 249-252} The coefficient of correlation for the whole series of 128 cases was found to be 0.74. When the cases were divided into groups according to low, normal, and high serum protein content by the micro-Kjeldahl method, the following values for the coefficient of correlation were obtained: In forty cases having total proteins between 3.45 and 5.50 Gm. per 100 c.c. of serum, the correlation coefficient for the two methods was 0.51; in seventy-nine with total protein content ranging from 5.50 to 7.00 Gm. per 100 c.c. of serum, the correlation coefficient was 0.45; and in nine in which the total protein was higher than 7.00 Gm. per 100 c.c. of serum, the coefficient of correlation was found to be 0.52. Apparently correlation between the two methods did not vary greatly with the total protein content of the serum. It is to be pointed out that the last group of nine cases is too small to be of statistical value.

Inspection of the albumin to globulin ratio in 125 of the 128 cases studied suggests that either an elevated globulin or a decreased albumin may have some effect on the serum specific gravity. The cases were divided into groups according to their albumin and globulin content as determined by the micro-Kjeldahl method. An albumin content above 3.0 Gm. and a globulin content below 3.0 Gm. per 100 c.c. of serum were regarded as normal. Within each of these groups, the total protein values as determined by the two methods were examined for differences not exceeding ± 0.3 Gm. per 100 c.c. of serum, which is the degree of accuracy found by Phillips and associates⁵ for the copper sulfate method. In 45 per cent of the cases in which the albumin concentration was within normal limits, the total protein as calculated from the specific gravity did not vary significantly from the results obtained by the micro-Kjeldahl determination. When the globulin content was normal, the results obtained by the two methods agreed closely in 48 per cent of the cases. These figures are similar to those found for the entire series of 125

cases in which the albumin and globulin were determined. In 44 per cent of the 125 cases, there were no significant variations between the total protein values found by the two methods.

In the group of sera having albumin concentrations below 3.0 Gm. per 100 c.c., the specific gravity method agreed with the micro-Kjeldahl in only 38 per cent of the cases. In all but one of the other cases of this group, the total protein calculated from the specific gravity was higher than that found by the micro-Kjeldahl determinations. When the globulin content was above 3.0 Gm. per 100 c.c. of serum, close agreement between the two methods was found in only 31 per cent of the cases. The remaining cases of this group were found to be almost equally divided between those with increased specific gravity and those in which the specific gravity was decreased.

Determinations of other blood constituents were not followed carefully enough in most of the cases in this group of 128 determinations to shed any light on the reasons for the observed variations between the Kjeldahl and specific gravity methods.

Most of the patients were hospitalized because of burns. A few of the determinations were done soon after the injury, but more of them were on patients who had been in the hospital for long periods of time and were being followed because of interest in wound healing and nutrition. In the hope of finding some biochemical conditions which might have had some consistent effect on the specific gravity of serum or plasma, the records of a larger group of patients were studied with particular reference to low serum electrolyte content, the presence of shock, and hemoconcentration. In most of these additional cases, plasma had been used instead of serum for the protein determinations.

In nine blood samples showing a low sodium content, the total protein as calculated from the specific gravity method did not differ from that obtained by the micro-Kjeldahl by more than ± 0.2 Gm. per 100 c.c. of plasma in three instances. Two comparisons showed a higher protein as estimated from the specific gravity, the difference being $+0.26$ and $+0.89$ Gm. per 100 c.c. of plasma, respectively. In four instances, the specific gravity method gave lower values, and the differences were -0.21 , -0.36 , -0.45 , and -1.00 Gm. of protein per 100 c.c. of plasma.

Serum chloride values lower than 99 meq./l were observed in seven samples of serum in which the protein was determined by both methods. The data are given in Table I. No data were available in which extremely low chloride values were found. A third sample of serum is included for Case 3 to show that there was still a wide variation between the protein values obtained by the two methods even though the serum chloride had become normal.

The plasma proteins of fifteen patients in shock were compared by both protein methods. In seven of the fifteen, the protein values by the two methods agreed with variations not greater than ± 0.2 Gm. per 100 c.c. of plasma. Four were found to have proteins higher by more than 1.00 Gm. per 100 c.c. of plasma by the specific gravity method than by the micro-Kjeldahl procedure. In the other four cases, the specific gravity method gave lower values, the differences varying from -0.37 to -1.56 Gm. of total protein per 100 c.c. of plasma.

TABLE I

CASE	DATE	TOTAL PROTEIN BY MICRO-KJELDAHL METHOD (GM. PER 100 C.C.)	TOTAL PROTEIN BY CuSO ₄ METHOD (GM. PER 100 C.C.)	VARIATION OF CuSO ₄ FROM KJELDAHL METHOD	CHLORIDE (MEQ./l)
1	10/27/44	6.54	6.17	-0.37	96.1
	10/28/44	5.89	6.93	+1.04	96.2
	10/29/44	6.34	8.00	+1.66	95.3
2	11/14/44	5.56	5.56	0	96.7
	11/15/44	5.64	5.40	-0.15	88.9
3	9/28/45	4.32	3.02	-1.30	88.5
	9/29/45	5.41	3.46	-1.95	94.5
	10/11/45	6.67	5.66	-1.01	104.6

Both methods for determining the total protein of plasma or serum were used in thirty-six patients in whom hemoconcentration was observed. As shown in Table II, the variations between values obtained by the two methods were distributed fairly evenly over a wide range, and it was impossible from this group of cases to draw any conclusions as to the possible effect of hemoconcentration on the specific gravity of plasma or serum.

TABLE II. PATIENTS WITH HEMOCONCENTRATION

DIFFERENCE BETWEEN TOTAL PROTEIN BY MICRO-KJELDAHL AND CuSO ₄ METHODS (GM. PER 100 C.C. PLASMA)	NUMBER OF CASES
CuSO ₄ , ± 0 to 0.20	5
CuSO ₄ , +0.21 to 0.40	6
CuSO ₄ , -0.21 to 0.40	4
CuSO ₄ , +0.41 to 0.60	3
CuSO ₄ , -0.41 to 0.60	3
CuSO ₄ , +0.61 to 0.80	2
CuSO ₄ , -0.61 to 0.80	3
CuSO ₄ , +0.81 to 1.00	2
CuSO ₄ , -0.81 to 1.00	3
CuSO ₄ , +more than 1.00	5
CuSO ₄ , -more than 1.00	0

If a larger number of cases were studied with analysis for other blood constituents and with more careful observations of the patient's condition, it might be possible to determine under what conditions it is safe to rely on the specific gravity as a means of calculating the serum protein. However, from our observations of many random cases in which the protein content was estimated by both the micro-Kjeldahl and by the copper sulfate specific gravity method, it was impossible to draw any conclusions regarding the causes for variations between the two methods. It is obvious from Figs. 1 and 2, which present a series of determinations in which meticulous laboratory technique was used, that it is unsafe to place too much confidence in serum protein values as estimated from the specific gravity when accurate determinations of protein are required.

SUMMARY

1. One hundred and twenty-eight cases are presented in which the serum protein concentration was determined by both the micro-Kjeldahl procedure and by the copper sulfate specific gravity method.

2. In fifty-five of the 128 cases, or 44 per cent, the differences between the total protein values by the two methods did not exceed 0.3 Gm. per 100 c.c. of serum. The percentage of cases showing such agreement was slightly lower when the albumin was below normal or the globulin above normal. When 0.2 Gm. per 100 c.c. is taken as the criterion for duplication, only 35 per cent of the cases showed such agreement.

3. No conclusions could be drawn as to the type of patient in whom the serum specific gravity is influenced by factors other than the protein content.

4. The correlation between the specific gravity of serum and its protein content was found to be too low to permit the use of specific gravity determinations for reliable estimation of serum total protein.

The authors wish to thank Miss Dorothy Keller and Miss Jeanette Maioli, for doing many of the nitrogen and specific gravity determinations.

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STUDIES ON STREPTOMYCIN*

I. ASSAY IN BODY FLUIDS

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VARIOUS methods of assaying antibiotics described in the literature are for the most part modifications of the cylinder-plate method of Abraham and associates¹ introduced as improvements on the original method. This is particularly true of penicillin. Foster and Woodruff² summarized and discussed the principles, merits, and disadvantages of different bacteriologic methods for the quantitative determination of penicillin and recommended a modification of the Oxford cup method for routine assays. According to Schmidt and Moyer,³ agar depth, stock culture of the test organism, pH of the medium, and other factors influence results obtained when the cup method is used. A stock suspension of *Bacillus subtilis* spores was recommended in preference to *Staphylococcus aureus* for penicillin assay by Foster and Woodruff.⁴ Similarly, *B. subtilis* was employed by these authors for streptothricin assay.⁵ For convenience and economy, large Pyrex baking dishes were used by Epstein and co-workers,⁶ while Beadle, Mitchell, and Bonner⁷ found large, rectangular culture plates advantageous for speed and consistency in routine assay work.

Simplifications of the procedure for applying the assay material to the agar surface have been reported. Cholden⁸ used a rubber stopper technique to make depressions in the agar to hold the assay liquid. Filter paper disks, instead of cups, were used by Sherwood, Falco, and de Beer,⁹ Vincent and Vincent,¹⁰ and Epstein and co-workers.⁶

Different streptomycin assay methods have been suggested and described. The turbidimetric method, the serial dilution procedure with *Escherichia coli* as the test organism, and the cylinder-plate technique using *B. subtilis* cells or spores were suggested by Waksman, Bugie, and Schatz.¹¹ Heilman¹² recommended a modification of Fleming's slide-cell technique for estimating the concentration of streptomycin in body fluids. This method was considered inferior to the cup-plate method for urine assay. To determine the degree of inactivation of streptomycin and streptothricin by cysteine, Denkelwater, Cook, and Teshler¹³ employed the cup method for assay. Using as a test organism a strain of *Staph. aureus*, Stebbins and Robinson¹⁴ found the cylinder-plate procedure satisfactory for the quantitative determination of streptomycin in the blood.

This investigation was conducted at Camp Detrick, Frederick, Md., from December, 1944, to October, 1945.

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*Streptomycin as referred to in this paper is streptomycin hydrochloride.

In the present report, a method is described for assaying streptomycin in body fluids, particularly in blood and urine. This method was developed for investigating the absorption, excretion, and activity of the drug in human beings and in laboratory animals, and it appears to be accurate and convenient for assaying numerous samples. The results of the procedure are based on the relationship between the diameter of the zone of inhibition and the amount of streptomycin diffusing into an agar plate seeded with a sensitive variant strain of *B. subtilis* Cohn *emend.* Prasmowski. Special assay agar was used.*

EXPERIMENTAL METHODS

Preparation of Spore Suspension.—

Strain: A variant strain of *B. subtilis* Cohn *emend.* Prasmowski, designated in this laboratory as the I strain, was chosen for the standard assay organism. It was preferred to several other organisms for the following reasons: (1) It sporulated readily on media used for spore production. (2) The viability of the spores was maintained better under the conditions of storage. (3) The zones of streptomycin inhibition were clearer and more defined. (4) The inhibitory action of normal blood observed when using *B. subtilis* var. *viscosus* Chester, has not been observed to date with human, rabbit, guinea pig, mouse, and dog blood, whether normal or infected.

Preparation of Inoculum for Spore Suspension: A pure culture was obtained by streaking the organisms (previously subcultured several times in dextrose broth) on dextrose agar, incubating at 37° C. for twenty-four hours, and transferring several rough, granular colonies onto slants. The slants were incubated for forty-eight hours at 37° C., and from them a suspension was made and used for inoculating large dextrose agar slants in thirty-two ounce prescription bottles. These were incubated for forty-eight hours, and a 50 c.c. aqueous suspension of the organisms was made from each bottle for mass inoculation of the media to be used for spore production.

Sporulation: Five cubic centimeters of the suspension described were added aseptically to beef heart infusion dextrose agar† slants in thirty-two ounce prescription bottles, stoppered with cotton plugs. Each bottle was tilted several times to facilitate inoculation of the entire surface of the slant. The bottles were incubated at 37° C. for approximately ten days, during which time they were examined grossly for contamination and microscopically for degree of sporulation. It was observed that a grayish-white, dry appearance of the colony indicated sporulation.

*Now available as Difco E-2 Streptothricin Assay Agar, Difco Laboratories, Incorporated, Detroit, Mich.

†Beef heart infusion dextrose agar:

Beef heart infusion (dehydrated)	2.5%
(Beef heart infusion from 500 Gm. beef heart)	
(Bacto tryptose, 1.0%)	
(Sodium chloride, 0.5%)	
Bacto-peptone	1.0%
Bacto-agar	1.5%
Dextrose (chemically pure)	1.0%
Distilled water (q.s. add.)	1.000 c.c.
Preliminary pH, 7.3; final pH, 7.0	
Autoclave, 15 pounds per 20 minutes	

Removal and Washing of Spores: After sporulation was almost complete, approximately 90 per cent, the growth was removed from the agar by adding approximately 5 Gm. of sterile glass beads and 10 c.c. of sterile 1.0 per cent phosphate buffer, approximately pH 7.0, and by rotating the bottle carefully from side to side. (By adding 5 Gm. each of monobasic and dibasic potassium phosphate to 1 L. of distilled water, a 1 per cent buffer of the desired pH can be obtained.) The resulting slurry and beads were then transferred into a sterile Erlenmeyer flask, and the bottle containing the slant was rinsed twice with 5 c.c. of sterile diluent. The suspension was shaken thoroughly and filtered through a sterile Buchner funnel containing sterile cheesecloth to remove the beads and bits of agar. The slurry was then dispensed into sterile centrifuge tubes, thoroughly mixed with a convenient quantity of the 1 per cent phosphate buffer, and centrifuged. This process of washing and centrifuging was repeated three times, and the washed spores were then transferred to a container and stored at 2° C. A sample was removed for microorganism count, after which part of the suspension was diluted to give the desired microorganism concentration (preferably eighty million per cubic centimeter) and stored at 2° C.

A spore suspension, prepared in the manner described, has now been used successfully for ten months. Thus, small laboratories can be spared the burden of producing spore suspensions, since a standardized product can be prepared in a central laboratory and dispensed as needed. Furthermore, recent experiments indicate that the spore suspension can be lyophilized with practically no loss in viability of the spores, facilitating ease of shipping.

Assay Technique.—

Preparation of Seeded Plates: For assaying, 200 c.c. of agar* were melted, cooled, and maintained at from 49 to 50° C. Prior to assay, the agar was seeded with *B. subtilis* spores (approximately 400,000 spores per cubic centimeter of agar) and maintained at 50° C. until all of the air bubbles emerged to the surface. Portions of 25 c.c. inoculated agar were transferred to large, flat-bottomed Petri plates (150 by 20 mm.), using a 30 c.c. glass syringe, equipped with an automatic syringe filler to facilitate rapid pouring. Seeded plates were allowed to cool and harden for ten minutes at room temperature. They were then dried uncovered at 50° C. for ten minutes to remove condensed moisture and allowed to return to room temperature for ten minutes with covers partly open.

It is essential that plating be performed on a level table to obtain a uniform depth of agar and that air bubbles disappear from the agar before it hardens. All timing should be carefully checked.

Plates prepared for assay can be stored at 2° C. for future use. However, there is a loss of accuracy proportional to length of storage time. Such plates must be redried at 50° C. and then allowed to attain room temperature before use.

Addition of Assay Material: Each plate selected for assay was superimposed over an area marked on a level table so that assay disk pad† could be placed in

*Difco E-2 Streptothricin Assay Agar.

†Schleicher and Schuell 740E one-half inch analytical filter pads were used for assay disks.

the same relative position on each consecutive plate (see Fig. 1). The material to be assayed was drawn into a 1 c.c. tuberculin syringe, graduated in hundredths, carrying a 26-gauge, 1-inch needle. The syringe was inverted and the air bubbles expelled, except in the assay of very dark fluids, where the retention of a few small air bubbles aided materially in establishing a sharp line of demarcation at the base of the plunger. Using forceps, an assay pad was carefully dropped onto the agar so that the smooth side of the pad was in contact with the agar surface. Precisely 0.15 c.c. of fluid was then *immediately* ejected from the syringe onto the pad. (In order to deliver 0.15 c.c. of assay material accurately, it is necessary that the barrel of the syringe be grasped between the thumb and forefinger of one hand and that the plunger be operated with a slight twirling motion with the thumb and forefinger of the other hand.) This

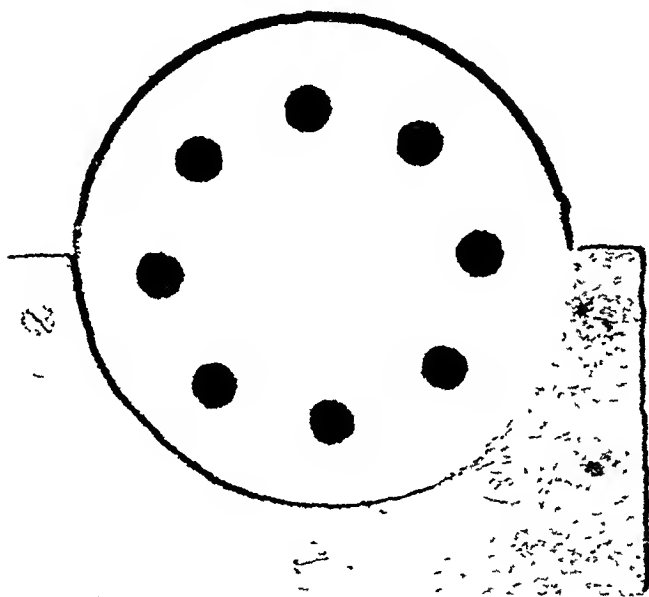


FIG. 1—The pattern used for superimposing seeded agar plates in order to place assay pads on the same relative position on each plate.

procedure was carried out in quintuplet for all samples assayed, one pad on each of five plates. Using this procedure, samples of assay material sufficient to assay a series of five plates previously poured and prepared can be completed in from fifteen to twenty minutes. A team of two persons is recommended for efficient assay operation with this procedure. However, one individual can successfully complete the operation.

Incubation and Reading of Assay Plates: The assay plates were incubated for seventeen hours at from 28 to 30° C., removed from the incubator, and the diameter of the zone of inhibition of growth measured (see Fig. 2). (Although assays could be completed in eight hours, a seventeen-hour incubation period was adopted for convenience.) Readings were recorded to the nearest 0.1 mm.

Values should be within plus or minus 1.0 mm. of one another. For example, if five readings for any hypothetical value are 21.0, 21.6, 20.9, 21.4, and 22.7, the last value (22.7) should be discarded. The average reading of the diameters of the zone of inhibition for each standard was plotted on graph paper against units per cubic centimeter for the respective standards. The diameter of the inhibition zone for any unknown assay sample was compared with the standard curve to give results in units of streptomycin per cubic centimeter of fluid.

Any plate-counting apparatus can be used for reading the inhibition zones, provided it is equipped with a transparent graduated film ruled in 1 mm. areas. The diameter can also be read with a millimeter rule. However, it

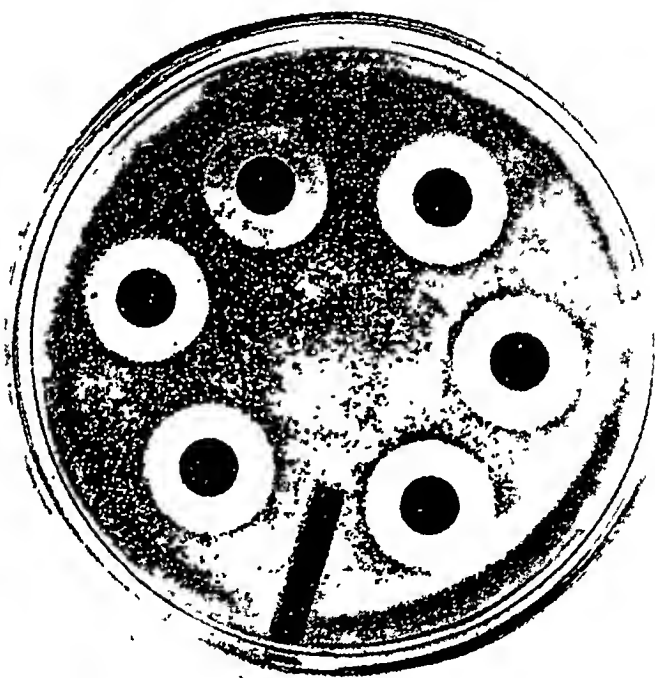


Fig. 2.—Illustrating the type of inhibition zone produced by streptomycin, using the pad method of assay.

was found convenient to use a positive exposed negative of 1 mm. graph paper on lithographic film.

Preparation of Standard Curves: Prior to treatment, a sample of body fluid, that is, blood or urine, was obtained for preparing a streptomycin standard curve. To aliquots of the selected fluid, varying concentrations of streptomycin were added and then assayed as previously described. The diameter of the resulting zone of inhibition was then plotted on graph paper against the corresponding unitage (see Figs. 3, 4, and 4A). For example, in following the blood level of streptomycin in a patient, a sample of blood was taken before the administration of the drug for constructing a standard curve on blood of that

particular person. Blood was collected in lithium oxalate (0.06 e.e. of a 3.3 per cent lithium oxalate solution per 1 e.e. of blood) and mixed. Then 0.9 e.e. of blood was accurately pipetted into sterile tubes, and to each of these 0.1 e.e. of streptomycin solutions of given variable unitage was added. (In pipetting blood for preparing standards, the outer surface of the pipette must be wiped to remove excess blood after it has been filled and before delivery to the test tube, and blood must be allowed to flow slowly from the pipette into the test tube from the 0 to the 0.9 mark.) A blank was used as a check to determine possible normal *B. subtilis* inhibiting properties of the blood. Water standards of streptomycin were prepared so that when 0.1 e.e. was added to 0.9 e.e. of blood or other liquid, concentration of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10 units of

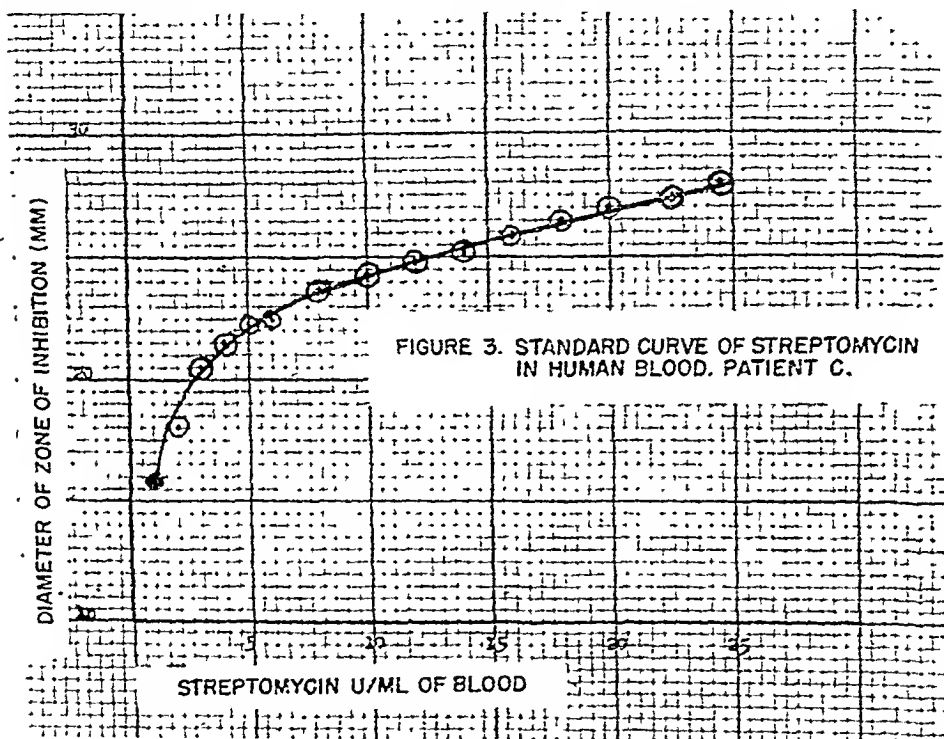


FIGURE 3. STANDARD CURVE OF STREPTOMYCIN IN HUMAN BLOOD, PATIENT C.

FIG. 3.

streptomycin per cubic centimeter resulted for the low unitage curve, and up through 30 units per cubic centimeter in steps of 2 units for the high unitage curve. (Standard solutions in water must be made so that the resulting unitages are 5, 10, 20, 30, 40, etc. Thus, when 0.1 e.e. of each standard is added to 0.9 e.e. of blood, the resulting unitage is decreased by one decimal.) Streptomycin standards from 0 through 6 units were placed on one series of five plates; standards from 8 through 18 and 20 through 30 were placed on two other series, respectively.

It is recommended that until experience is gained with the method a complete standard curve should be run from 0 to 30 units per cubic centimeter.

This will necessitate obtaining a 20 c.c. sample of blood from the patient before administration of streptomycin. From experience with correlating blood levels and dosages, one can soon learn to select that portion of the curve that will be required to determine blood levels from a given dose. This will permit the use of a smaller blood sample for making the standard curve. Only a 1 c.c. sample of blood is required for determining the blood level of a patient in treatment.

Fig. 4.

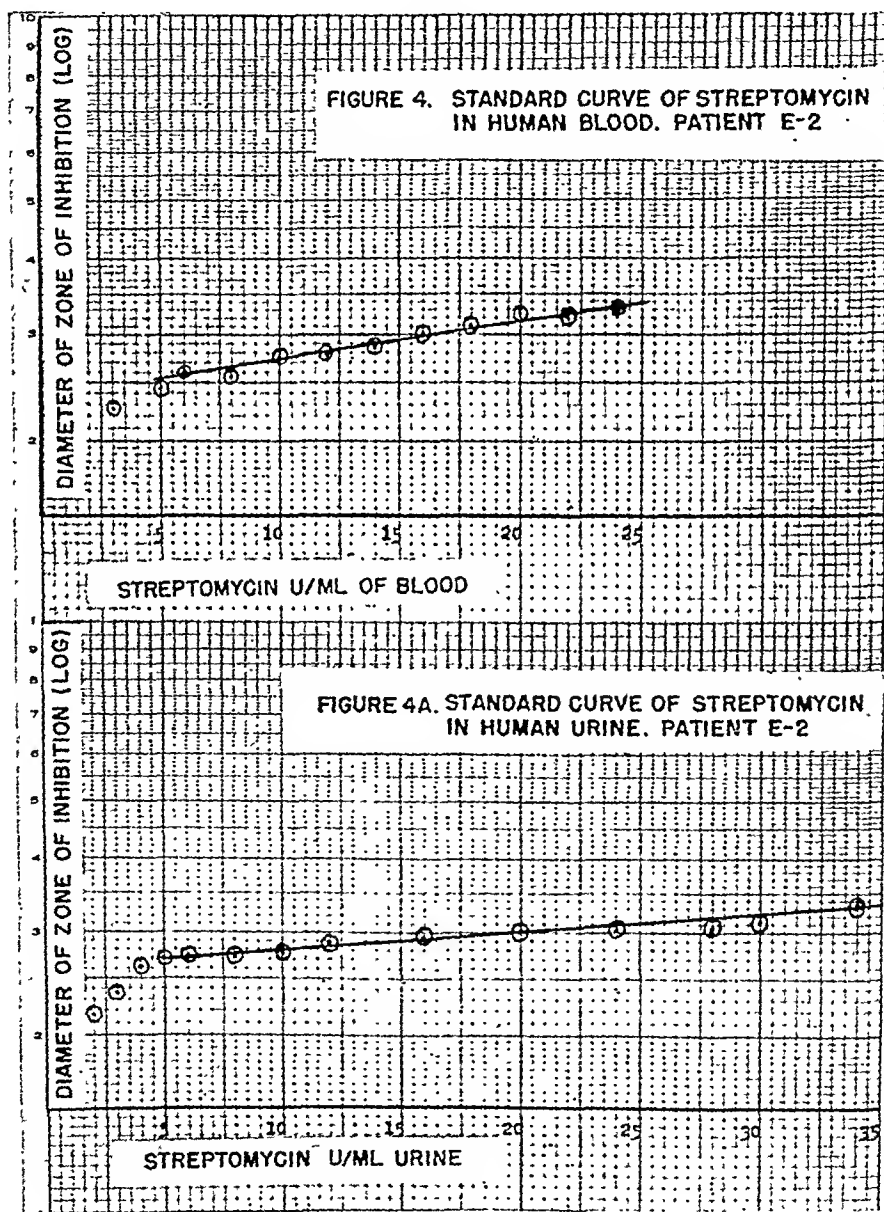


Fig. 4A.

TABLE I. VARIATIONS IN INDIVIDUAL STANDARD CURVES

STREPTOMYCIN ADDED (UNITS PER C.C.)	DIAMETER OF ZONE OF INHIBITION (MM.)			
	PATIENT D	PATIENT E	PATIENT F	PATIENT G
0	0	0	0	0
0.5	15.8	16.0	16.4	0
1	18.4	20.0	18.2	15.8
2	20.4	21.1	22.5	18.1
3	21.5	21.7	24.3	20.5
4	21.8	22.4	-----	21.6
5	22.0	22.6	22.5	22.3
6	-----	23.4	26.2	22.4
8	22.4	24.9	27.4	23.6
10	22.6	25.5	28.4	24.2
12			29.7	24.7
14			30.2	25.2
16			30.4	25.7
18			30.6	26.4
20			31.0	26.8
22			31.9	27.4
24			31.6	27.8
26			32.5	

Streptomycin levels in urine were determined by the same procedure as for blood. When high levels were anticipated (above 25 units per c.c.), the urine was diluted with water.

Data for standard curves for four patients treated with streptomycin are presented to illustrate the assay method and to demonstrate the necessity for individual standard curves (see Table I). Fig. 3 shows a typical standard curve. The semilogarithm standard curves on the blood and urine of Patient E-2 (Fig. 4 and 4A) were used to estimate the very high drug levels. Although some work was completed dealing with the dilution of bloods containing high unitages of streptomycin (40 units per cubic centimeter and above), sufficient data were not accumulated to warrant recommendations. Results plotted on semilogarithm paper show the curve to be a straight line function, and it is reasonable to assume that, for all practical purposes, such high unitages can be determined.

APPLICATION OF ASSAY TECHNIQUE

Application of this method for determining streptomycin levels in man and in various laboratory animals is discussed in a succeeding report.¹² This method has also been applied successfully to the assay of streptothricin and other antibiotics. This method for streptomycin assay was adopted by the Office of the Surgeon General, United States Army, on September, 1945, for use in Army hospitals.

SUMMARY

A method for the quantitative determination of streptomycin and other antibiotics in body fluids has been described in detail.

The authors wish to express their gratitude to Prof. H. E. Carter and associates, for permission to use the original formula for streptothricin assay agar; to Merck & Company, Inc., New York, N. Y., for the supply of streptomycin; to Anna E. Funk, for suggesting the use of an automatic syringe filler; and to Lieutenant Colonel Murray Sanders, for his kind suggestions and careful guidance.

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STUDIES ON STREPTOMYCIN*

II. BLOOD LEVELS AND URINARY EXCRETION IN MAN AND ANIMALS

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IN INVESTIGATIONS of the chemotherapeutic activity of streptomycin, both in laboratory animals and man, basic information was needed concerning absorption of the drug by various routes and its distribution and excretion in several species of animals. At the inauguration of the experiments herein reported, a limited amount of information, chiefly toxicologic, was provided by Merck Institute for Therapeutic Research.¹ More recently, data of other investigators have been reported in the literature.^{2, 3} A limited amount of additional information on the behavior of the drug in man is available but has not yet reached general circulation.⁴

A study of streptomycin in a variety of animal species was carried out preliminary to, or during, experiments designed to test therapeutic effectiveness of the antibiotic in specific bacterial infections. In addition, the use of the drug for treatment of infection in hospitalized patients provided an opportunity for comparison of results of animal experiments with the findings in man. However, for present purposes, data relative to the effect of the antibiotic on the infectious diseases have been excluded from this report. In all cases, whole blood, with an oxalate anticoagulant, was used for streptomycin determinations performed according to the method previously described.⁵

PROCEDURE AND RESULTS

Parenteral Administration.—Streptomycin blood levels were determined in the mouse, guinea pig, rabbit, monkey, and man. The drug was administered by subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal injection.

Mice: Animals were weighed to the nearest 0.5 gram, and graded doses of streptomycin in sterile distilled water were administered with a tuberculin syringe. The concentration of streptomycin solution was adjusted so that doses of approximately constant volume were given. Following administration, blood samples were obtained by heart puncture from the etherized or chloroform-anesthetized animal, and at the same time extracts of the liver, spleen, and kidneys were made by trituration of each organ with sterile water and sand followed

*These studies were conducted at Camp Detrick, Md., from December, 1944, to October, 1945.
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*Streptomycin as referred to in this paper is streptomycin hydrochloride and was supplied by Merck & Company, Inc., New York, N. Y.

TABLE I. STREPTOMYCIN LEVELS IN BLOOD AND TISSUE EXTRACTS OF MICE* FOLLOWING INTRAMUSCULAR INJECTION OF THE DRUG

TIME (HOURS)	DOSE, 5,000 UNITS PER KG.				DOSE, 10,000 UNITS PER KG.			
	BLOOD LEVEL (UNITS PER C.C.)		KIDNEY (UNITS PER ORGAN)		BLOOD LEVEL (UNITS PER C.C.)		KIDNEY (UNITS PER ORGAN)	
	EXP. 1	EXP. 2	EXP. 1	EXP. 2	EXP. 1	EXP. 2	EXP. 1	EXP. 2
0	0	0	0	0	0	0	0	0
1/6	>10	8	5.0	<2	8	8.5	<0.5	<2
	>10	9	<0.5	<2	8	6.5	<0.5	<2
1/3	>10	7.5	<0.5	<2	8	8.5	<0.5	<2
	>10	6.5	<0.5	<2	8	6.5	<0.5	<2
1/2	>10	6.5	<0.5	<2	6.5	8.0	<0.5	<2
	>10	6.5	<0.5	0	6.5	6.5	<0.5	<2
3/4	6.5	1.5	0	<2	2.0	6.5	<0.5	<2
	4.0	1.5	<0.5	0	2.5		<0.5	<2
1	5.0	5.0	<0.5	<2	4.0	3.5	<0.5	<2
	3.0	0.5	0	<2	6.0	3.0	<0.5	<2
1 1/4	3.0	2.0	0	0	2.0	1.0	0	<2
	3.0	0.5	0	0	<0.5	3.0	0	<2
1 1/2	<2.5	0.5	0	0	2.5	2.0	0	0
	2	<0.5	0	0	<0.5	0.5	<0.5	<2
1 3/4	2.5	<0.5	0	0	<0.5	<0.5	0	<2
	<2	<0.5	0	0	0	1.5	0	<2
2	<2	<0.5	<0.5	0	<0.5	0	0	0
	<2	0	0	0	0	0	0	<2
2 1/4	<2	0	0	0	<0.5	0	0	<2
	<2	0	0	0	<0.5	0	<0.5	<2
2 1/2	0		0	0		0		<2
	0		0	0	0			<2
2 3/4	0	0	0	0	<0.5		0	<2
	0	0	0	0	<0.5		0	<2
3	0	0	0	0	<0.5	0	0	<2
	0	0	0	0	<0.5	0	0	<2
3 1/2	0			<2		0		<2
	0			<2		0		<2
4	0			0		0		<2
	0			0		0		<2
4 1/2	0			0		0		<2
	0			0		0		<2
5	0			<2		0		<2
	0			0		0		<2
5 1/2	0			0		0		<2
	0			0		0		<2
6	0			0		0		<2
	0			0		0		<2
	0			<2		0		<2

*Extracts of liver and spleens negative in every case.

by centrifugation (1 c.c. water used for spleen and kidney; 2 c.c., for liver). Two animals were sacrificed for each time interval, and separate determinations were made. The tissue extracts were assayed against standard curves of normal tissue extract supernatants of like concentration.

The streptomycin levels in the blood and tissue extracts of mice, following intramuscular administration of the drug, are given in Table I. From Table I it can be seen that doses of 5,000 and 10,000 units per kilogram of streptomycin in mice produced a maximum blood level of 8 units per cubic centimeter, or above, and that the blood concentration of the drug dropped rapidly and disappeared about two hours after the injection. Of the organs assayed, the liver and spleen were negative at all times, and the kidney concentration was so low as to indicate that the drug was merely in a state preparatory to elimination.

TABLE II. BLOOD LEVELS OF STREPTOMYCIN IN GUINEA PIGS AFTER SINGLE DOSE ADMINISTRATION

METHOD OF ADMINISTRATION	I.D.	I.P.	S.C.	S.C.	I.D.	I.P.	I.M.	S.C.	S.C.	S.C.	S.C.
DOSE (UNITS PER KILOGRAM)	2500	2500	3000	5000	5000	5000	10,000	15,000	17,500	20,000	22,500
TIME (HOURS)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)	BLOOD LEVEL (UNITS PER C.C.)
0	0	0		5	<5	0					
1/4	2.0	1.0			0.5		5.5				
							5.5				
							8.5				
1/2	3.0	2.0	6.5	1.2	3.0	3.0	6.5	17.0	20.0	20.0	18.5
			5.5				6.0	15.0		17.0	19.5
			4.5				10.0	12.0		14.0	
3/4	4.0				3.0	3.5	6.7				
							6.0				
							8.5				
1		3.0	5.2	4.7	4.5	4.0	5.5	21.0	26.0	20.5	22.5
			5.7				7.0	15.0		21.5	26.5
			6.5				9.0			15.0	
1 1/4	4.0								20.5	23.0	26.0
							7.5	19.0		23.0	23.0
							>10.0			20.5	
1 1/2	2.5	2.5	4.7	4.7	6.5	4.5	6.5	21.7	25.0	21.5	26.5
			4.7				6.5	15.0		23.5	26.5
			7.2				>10.0			17.0	
2	3.5		4.5	3.7		3.5	8.0	16.3	20.5	23.0	23.0
			4.5				7.0	11.5		21.5	
			7.2					16.0		19.0	
2 1/4					5.5		8.0				
							8.0	18.5		19.0	
								16.0		23.0	
2 1/2				5.5		1.0	7.5				
							7.0	16.0		19.5	
										20.5	
3	3.0		4.5	4.0	5.0		5.5				
			3.2								
			4.7								
3 1/2	3.0	1.5	2.5	2.5			7.5	13.5			
			3.2								
			7.0								
4	3.0	1.0	3.5	2.7		2.5	7.5				
			2.2								
4 1/2	3.0	1.0	2.5	3.2		1.5	6.5				
			2.2								
			3.2								
5			2.7	4.0		6.5					
			3.7								
			5.5								
5 1/2			4.5	2.5			5.5				
			4.7								
			2.7								
6	1.5		4.0				6.0				
			5.0								

I.D., Intradermal; I.P., Intraperitoneal; S.C., Subcutaneous; I.M., Intramuscular.
 Lungs, spleen, and liver extracts prepared and found negative. Kidney extracts positive to extent of approximately from 5 to 15 units per organ.

Guinea Pigs: Animals were weighed to the nearest 5 grams, and streptomycin in sterile distilled water or sterile 0.85 per cent saline solution was administered by tuberculin syringe. Blood samples for assay were obtained at varying intervals by heart puncture, generally under pentobarbital sodium anesthesia, which was found not to affect the test. Cheek runs were made without anesthesia. In order to maintain blood volume, 5 per cent glucose in 0.85 per cent saline solution was given to the animals at periodic intervals. When using large animals, individual standard curves were prepared for each, but this proved undesirable for smaller guinea pigs due to blood depletion. For such animals, a standard curve of pooled guinea pig blood was prepared and used for several animals. Blood levels of streptomycin in guinea pigs, following administration of graded doses of the drug by various routes, are given in Table II. These results indicate that the rate of absorption of streptomycin varied with the route of administration. Variations in blood levels were observed with changes in dosage, but the relationship was not directly proportional. The drug was found in the blood as late as six hours after injection.

TABLE III. TYPICAL BLOOD LEVELS IN RABBITS FOLLOWING INTRAMUSCULAR INJECTION OF 10,000 UNITS PER KILOGRAM OF STREPTOMYCIN

RABBIT 3A81			
HOURS AFTER INJECTION	STREPTOMYCIN BLOOD LEVEL* (UNITS PER C.C.)	HOURS AFTER INJECTION	STREPTOMYCIN BLOOD LEVEL (UNITS PER C.C.)
0.5	>10	3.5	4.5
1	>10	4	4.0
1.5	>10	4.5	3.5
2	6.2	5	4.0
2.5	5	5.5	2.5
3	4	6	1.5

*Blood level determined in oxalated whole blood.

Rabbits: Although the rabbit has been the chief source of supply for blood when checking standard curves for various changes in the assay method, comparatively few data have been obtained on correlation of blood level with dosages of streptomycin. However, the available data indicate that the rabbit reacts to streptomycin more like the guinea pig than the mouse. Thus, it appears to be able to retain the drug sufficiently to permit administration at from three- to four-hour intervals. Typical streptomycin blood levels obtained in a rabbit following intramuscular injection of 10,000 units per kilogram are given in Table III and were found to range from above 10 units per cubic centimeter at one-half hour to 1.5 units per cubic centimeter at six hours. Blood samples for analysis were obtained from the marginal ear vein.

Monkeys: Two animals were injected intramuscularly with 1,250 units per kilogram of streptomycin. The blood of one of these animals was assayed for streptomycin levels at frequent intervals for three hours, and the second animal was studied for six hours. Results are given in Table IV and indicate that the monkey blood level ranged from 2.5 units per cubic centimeter at fifteen minutes to less than 0.5 unit per cubic centimeter at five hours after the injection, with later samples being negative.

TABLE IV. BLOOD LEVELS IN TWO MONKEYS FOLLOWING INTRAMUSCULAR INJECTION OF 1,250 UNITS PER KILOGRAM OF STREPTOMYCIN

HOURS AFTER INJECTION	STREPTOMYCIN BLOOD LEVELS*	
	MONKEY 49	MONKEY 50
0.25	†	2.7
0.5		2.5
0.75		2.2
1	1.25	1.7
1.5	0.75	1.5
2	0.5	1.0
2.5	0.4	1.0
3	0.4	0.7
3.5	0.4	
4	0.4	
4.5	0.4	
5	0.4	
6	0	

*Blood level determinations made on oxalated whole blood.

†Determination not made.

TABLE V. AVERAGE (SIX PADS) ZONE INHIBITION OF STREPTOMYCIN IN BLOOD OF EXPERIMENTALLY INFECTED MONKEYS; FIGURES FOR PLOTTING INDIVIDUAL MONKEY STANDARD CURVES (MM.)

STREPTOMYCIN ADDED (UNITS PER C.C.)	DIAMETER ZONE INHIBITION (MM.)						
	MONKEY						
	49	57	61	63	66	67	71
0	0	0	0	0	0	0	0
0.2	T*	16.5	16.7	16.8	16.6	T	T
0.5	17.4	17.9	19.2		18.7	17.7	17.4
1.0	20.3	20.1	20.5	21.3	19.8	19.7	19.6
2.0	21.9	†	21.3		21.5	21.4	21.7
3.0	22.7	23.4	22.2	23.9	22.0	22.7	23.0
4.0	23.4		22.5		22.8		23.5
5.0	23.7	24.1		24.9	23.5	24.1	24.0
6.0	24.3		23.7		23.8		24.5
8.0	24.8	24.6		25.4	24.0		

*T. Trace quantity.

†Number of points determined for standard curve limited by quantity of blood available.

Following these experiments, streptomycin blood levels were followed in seven monkeys, five of which had been experimentally infected, the remainder serving as drug controls. The same dose as mentioned, that is, 1,250 units per kilogram, was given every three hours for nine days by intramuscular injection. In Table V are presented the data used for establishing the individual standard curves for each monkey and the individual variability in reaction between blood and streptomycin is shown. In Table VI are shown the daily blood levels of the animals and in Table VII, the rate of disappearance of the drug from the blood stream of individual animals after the last injection. Additional evidence of the individual reaction of animals to streptomycin with variation in daily blood levels, as well as in the rate of disappearance of the drug, is also shown in Tables VI and VII.

Man: In the preliminary studies several standard curves were made for normal human beings, but the blood level of streptomycin from parenteral administration was followed in only four patients, and urinary excretion was determined on two of these patients. In Table VIII is summarized the strepto-

TABLE VI. BLOOD LEVELS OF EXPERIMENTALLY INFECTED AND CONTROL MONKEYS RECEIVING STREPTOMYCIN THERAPY* (TREATMENT, STREPTOMYCIN 1,250 UNITS PER KILOGRAM INTRAMUSCULARLY EVERY THREE HOURS FOR NINE DAYS; DETERMINATIONS MADE FOLLOWING 8 A.M. INJECTION EXCEPT AS NOTED)

DAY OF THERAPY	MONKEY 49†			MONKEY 61†			MONKEY 71			MONKEY 63			MONKEY 57			MONKEY 66			MONKEY 67		
	TIME AFTER IN- JECTION		UNITS PER C.C.	TIME AFTER IN- JECTION		UNITS PER C.C.	TIME AFTER IN- JECTION		UNITS PER C.C.	TIME AFTER IN- JECTION		UNITS PER C.C.	TIME AFTER IN- JECTION		UNITS PER C.C.	TIME AFTER IN- JECTION		UNITS PER C.C.			
	HR.	MIN.		HR.	MIN.		HR.	MIN.		HR.	MIN.		HR.	MIN.		HR.	MIN.		HR.	MIN.	HR.
5/13/45	7	3.8	19	>6(7.0)†	2	35	2.6	43	1.6	54	4.0	1	1	3.1	1	10	2				
1	2	16	2.3	2	24	2	30	1.9	2	45	1.2	2	53	1.5	3	10½	1				
5/14/45																					
2	10	3.9	20	>6(7.7)	26	4.2	42	4.1	4.1	50	4.4	1	6	3.7	1	15	3.7				
5/15/45	20	8.0	27	>6(10)	33	6.0	40	4.1	4.1	50	8.0	1	5	6.0	1	10	4.6				
3	2	20	3.3	2	37	3.6	2	2.4	2	32	2.1	2	45	1.7	2	55	3.2				
5/16/45																					
4	10	8.0	35	>6(10)	17	5.5	25	5.3	5.3	30	3.6	40	5.7	45	4.1	1.7	4.6				
5/17/45	1	9	4.2	34	5.5	14	4.0	20	2.5	30	2.7	40	6.8	1.5	3	55	4.6				
5	2	35	1.2	2	52	2.2	2	40	1.2	2	49	1.2	2	58	1.5	3	4.1				
5/18/45																					
6	5	3.2	11	>6(9.0)	28	3.0	40	2.3	2.3	17	3.5	55	3.3	1	5	2.7	2.7				
5/19/45	13	4.5	20	>6(9.0)	25	6(7.5)	31	2.3	2.3	40	3.4	48	5.0	1	1	4.3	4.3				
7	2	32½	0.9	2	37½	0.7	2	27½	2.0	2	43½	1.7	2	53½	1.1	3½	2.2				
5/20/45																					
8	10	7.1	15	>6(8.0)	22	3.5	30	2.6	2.6	37	3.6	47	4.1	55	3.7	4.1	3.7				
5/21/45	10	4.1	15	>6(8.0)	18	5.2	25	5.6	5.6	30	5.8	35	8(8.3)	42	4.2	4.2	4.2				
9	2	45	1.5	2	50	1.6	2	54	1.5	2	58	0.5	3	2½	1.0	3	1.6				

*Blood of all animals negative for *Bacillus subtilis* inhibition immediately prior to treatment.

†Uninfected animals.

‡Blood levels above highest point of standard curve: the figure in parenthesis represents best estimate.

§Time more than three hours due to from five to fifteen-minute period necessary for infection.

||Units per c.c. after 11:00 A.M. infection: remainder from 8:00 A.M. infection.

TABLE VII. RATE OF DISAPPEARANCE OF STREPTOMYCIN FROM BLOOD OF MONKEYS AFTER LAST INTRAMUSCULAR INJECTION OF DRUG; BLOOD LEVELS IN UNITS PER CUBIC CENTIMETER

MONKEY	TIME AFTER LAST INJECTION (HR.)						
	0.5	3.0	6.0	9.0	12	18	24
57	5.8	1.0	0.6	T*	0	0	0
61	>6 (8.0)	1.6	0.6	<0.5	T	T	T
63	5.6	0.5	<0.5	T	T	0	0
66	>8 (8.3)	0.7	T	T	0	0	0
67	4.2	1.6	0.7	0	0	0	0

*T, Trace quantity.

TABLE VIII. STREPTOMYCIN BLOOD LEVELS IN PATIENTS UNDER INTENSIVE THERAPY COURSE OF DRUG*

PATIENT ONE			PATIENT TWO			PATIENT THREE			PATIENT FOUR		
DAY OF TREATMENT	HOURS AFTER INJECTION	BLOOD LEVEL (UNITS PER C.C.)	DAY OF TREATMENT	HOURS AFTER INJECTION	BLOOD LEVEL (UNITS PER C.C.)	DAY OF TREATMENT	HOURS AFTER INJECTION	BLOOD LEVEL (UNITS PER C.C.)	DAY OF TREATMENT	HOURS AFTER INJECTION	BLOOD LEVEL (UNITS PER C.C.)
1	1½	1.2				1	1½	6.0	1	1½	1.1
	1½	2.0					2½	6.0		2½	1.9
	2½	1.0					3½	4.0		3½	2.6
	3½	1.1					1	8.5		1	2.0
	½	2.1									
2	3	1.2	2	3	1.3	2	3	8.0	2	3	2.3
	1	1.6		¼	2.1		1	9.0		¾	7.4
				½	3.1						
				¾	2.1						
				1	2.5						
				1½	1.6						
				3	1.0						
3	1	2.1	3	2½	1.1	3	3	10.5	3	3	3.6
	3	1.6		¾	1.0		1½	11.0		¾	7.4
	¾	2.0									
									4	3	2.0
										1½	3.6
5	3	1.0							5	2	8.5
	1	1.4								3	4.0
									6	3	3.1
										1	7.2
										3	4.8
										1	5.3
7	3	0.9							8	3	5.7
	5	0.7								5	2.8
	7	0.6								7	1.9
	9	<0.5								9	1.7
										28	Trace

*Courses of therapy were as follows:

Patient 1, treatment started 10 A.M., May 8, 1945; 30,000 units intramuscularly every three hours. Stopped May 14, 1945. Total dose, 1,440,000 units. Lot 181.

Patient 2, treatment started noon, April 18, 1945; 30,000 units intramuscularly every three hours. Stopped 6 P.M., April 21, 1945. Total dose, 810,000 units. Lot 4R307L.

Patient 3, treatment started 11 A.M., May 19, 1945; 250,000 units intramuscularly every three hours. May 23, 1945; 250,000 units intravenously noon and 3 P.M.; 500,000 drip; May 24, 1945, 500,000 units intravenously, drip, midnight. Then 1,000,000 drip every six hours to 8 P.M., May 26, 1945, at which time last dose of 500,000 units intravenously was given. Total dose, 13,750,000 units. Lot 212.

Patient 4, treatment started 10 A.M., May 21, 1945; 100,000 units intramuscularly every three hours. Stopped 7 A.M., May 28, 1945. Total dose, 5,600,000 units. Lot 212.

†Determinations following first dose of drug.

TABLE IX. DAILY URINARY EXCRETION OF STREPTOMYCIN BY TWO PATIENTS DURING AND AFTER INTENSIVE THERAPY*

DAY AFTER TREATMENT BEGUN	PATIENT 3		PATIENT 4	
	URINE VOL. 24 HR. (C.C.)	URINARY EXCRETION (UNITS)	URINE VOL. 24 HR. (C.C.)	URINARY EXCRETION (UNITS)
1	675	241,383	1,830	819,000
2	510	218,700	925†	509,083
3	940	677,980	1,300	552,933
4	1,700	744,600	1,550	888,066
5	2,950	1,923,400	1,825	773,219
6		‡	1,400	645,766
7	1,350	578,700	1,730	625,107
8	1,300§	180,266	1,700§	727,600
9	1,100	23,137	1,350	179,550
10	2,900	21,605	1,200	27,040
11	1,900	18,113	1,350	11,250
12	1,450	5,520	1,400	5,600
13	2,150	7,525	1,375	9,625
14	2,500	9,750	2,025	8,100
15	1,700	5,780	1,500	5,550
16	1,750	4,200	1,050	1,890
17	1,550	4,340	1,650	2,805
18	1,750	3,150	2,000	1,800
19		‡	2,100	2,205
20	1,850	3,700	1,500	1,350
21	2,020	4,243	1,800	2,340
22	1,350	1,350	1,575	T
23	1,400	2,520	1,375	T
24	2,000	1,200	1,425	T
25	2,000	1,600	850	T
26	2,000	‡		
27	2,300	7,130		
28	2,390	1,195		
29		0		
Total recovered		4,691,087		5,800,479
Total adm.		15,570,000		5,600,000

For course of therapy, see footnote of Table VIII.

†Twelve hour sample.

‡No determination made.

§Treatment stopped.

||Trace; impossible to read accurately due to urinary contamination of *Pseudomonas* sp. that was also inhibitory to *Bacillus subtilis*.

mycin blood levels of four patients under treatment, the clinical results of which will be reported elsewhere. Daily urinary excretions of two patients are given in Table IX. The results presented in Table VIII indicate that in human beings a dose of 30,000 units of streptomycin every three hours by intramuscular injection would provide a blood level of from 1 to 2.5 units per cubic centimeter and that the blood level can be increased by increasing the dose. In Table IX it is shown that large amounts of the drug are eliminated by the kidney, but with daily variations. Further, urinary contamination interferes with the accuracy of the method.

In order to check the assay methods statistically for correlation from human being to human being, the data used for establishing thirteen individual standard curves are presented in Table X, and a statistical analysis of these data for points up through 10 units per cubic centimeter is shown in Table XI. Although doses now used for therapy frequently result in blood levels higher than 10 units per cubic centimeter in human beings, the data presented indicate that the

TABLE X. DATA FOR PREPARATION OF STANDARD CURVE FOR THIRTEEN HUMAN BEINGS

PATIENT	QUANTITY STREPTOMYCIN ADDED TO WHOLE BLOOD (UNITS PER CUBIC CENTIMETER)									
	0.5	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0	
1	17.4	18.8	20.0	22.1	22.8	23.0	23.7	24.6	25.0	
2	16.3	18.4	19.9	20.9	21.5	22.2	23.0	23.4	23.3	
3	17.4	18.9	20.3	21.5	22.4	23.2	24.1	23.9	24.5	
4	18.7	19.9	21.4	22.1	*	22.7		23.8		
5	18.2	19.3	21.2	22.1	22.8	23.5	24.1			
6	16.6	17.9	20.2	21.2	22.0	23.3	23.1	23.5	24.6	
7	15.8	18.2	20.4	21.5	21.8	22.0		22.4	22.6	
8	Trace	19.3		22.7		24.4	26.1	25.4	27.2	
9	16.0	20.0	21.1	21.7	22.4	22.6	23.4	24.9	25.5	
10	18.0	21.1	23.4	24.5	25.5	25.1	25.4	25.3	26.4	
11	15.1	20.8	24.0	24.3	26.9	27.9	27.6	28.7		
12	16.4	18.2	22.5	24.3		25.5	26.2	27.4	28.4	
13	0	15.8	18.1	20.5	21.6	22.3	22.4	23.0	24.2	

*Determination not made.

TABLE XI. STATISTICAL ANALYSIS OF DATA USED IN ESTABLISHING STANDARD CURVES FOR THIRTEEN HUMAN BEINGS*

CONCENTRATION	NUMBER OF OBSERVATIONS	STANDARD DEVIATION	STANDARD ERROR OF MEANS	MEANS	MEANS MINUS 2 STANDARD ERRORS	MEANS PLUS 2 STANDARD ERRORS	COEFFICIENT OF VARIATION
0.5	11	1.154	0.348	16.945	16.249	17.641	6.81
1.0	13	1.377	0.332	18.969	18.205	19.733	7.26
2.0	12	1.632	0.471	21.042	20.100	21.984	7.76
3.0	13	1.328	0.368	22.262	21.526	22.998	5.96
4.0	10	1.792	0.567	22.970	21.836	24.104	7.80
5.0	13	1.672	0.464	23.669	22.741	24.597	7.06
6.0	11	1.632	0.492	24.464	23.480	25.448	6.67
8.0	12	1.833	0.529	24.692	23.634	25.750	7.42
10.0	10	1.764	0.558	25.170	24.054	26.286	7.01

*Courtesy of Ensign M. R. Zelle.

method is reliable, and to date standard curves through 24 units per cubic centimeter have been made without serious difficulty.

Oral Administration.—To test the absorption of streptomycin from the gastrointestinal tract, the drug was given orally to mice, rabbits, dogs, and man.

Mice: For oral administration to mice, a tuberculin syringe fitted with a long, smoothly blunt, 18-gauge needle was used. The drug was dissolved in sterile distilled water to a concentration such that a mouse weighing 20 grams would receive 0.5 c.c. Animals receiving the same quantity of saline by the oral route were sacrificed for controls. Blood samples were obtained by heart puncture of anesthetized animals 0.25, 0.5, 1.0, and 1.5 hours after administration. In no case was streptomycin detected in the blood of the twenty animals given the drug orally.

Rabbits: Two animals were given 10,000 units per kilogram of streptomycin mixed with oil of theobroma, U. S. P., and contained in gelatine capsules. For administration, a stomach tube of sufficient diameter to pass the capsule was used. Blood samples were obtained from the marginal ear vein at frequent intervals. At no time was a detectable quantity of streptomycin found in the blood of either rabbit.

TABLE XII. ORAL ADMINISTRATION OF STREPTOMYCIN IN DOGS USING VARIOUS VEHICLES;
NO SIGNIFICANT BLOOD LEVELS ATTAINED

DOG	SEX	WEIGHT (KG.)	DOSE (UNITS PER KG.)	OBSERVA- TION TIME (HR.)	VEHICLE FOR ADMINISTRATION OF DRUG
B	M	12	2,000	6	Oil of theobroma plus alcohol
L	F	20	6,250	6.5	Oil of theobroma plus alcohol
B	M	12	6,000	7	Oil of theobroma
L	F	20	6,000	7	Oil of theobroma
L	F	20	10,000	3	Amphojel,* 5 c.c., 15 min. before drug (drug as powder in gelatin capsule)
P	M	21	10,000	3.5	Amphojel,* 5 c.c., 15 min. before drug in oil of theobroma plus alcohol
L	F	20	10,000	5	Acacia emulsion (O/W) of corn oil, H ₂ O, alcohol
P	M	21	10,000	5	Drug dissolved in milk
J	M	8	10,000	5	H ₂ O containing 1 Gm. disodium phosphate
R	F	10	10,000	5	H ₂ O containing 1 Gm. sodium citrate
M	M	8	10,000	6	H ₂ O plus alcohol (1 c.c.)
G	F	8	10,000	6	H ₂ O plus alcohol (2 c.c.)
B	M	12	10,000	6	H ₂ O plus alcohol (3 c.c.)
J	M	8	10,000	7	Lanolin emulsion (W/O) of corn oil, water
G	F	8	10,000	7	Lanolin emulsion (W/O) of corn oil, water
R	F	10	10,000	6	Yolk of egg
L	F	20	10,000	6	White of egg
J	M	10	10,000	6	White of egg
G	F	10	10,000	6	Yolk of egg

*John Wyeth & Brother, Inc., Philadelphia, Pa.

Dogs: Seven dogs were employed to test the suitability of several vehicles for aiding absorption of streptomycin after oral administration, and the results are summarized in Table XII. In no case was a detectable quantity of streptomycin found in the blood, but random urine samples from untrained dogs showed detectable and often considerable urine levels of the drug.

Man: One volunteer was administered 500,000 units* (6,666 units per kilogram) of streptomycin orally in each of two doses three hours apart. The drug was mixed with oil of theobroma, U. S. P., and inclosed in gelatin capsules. Blood samples 0.5, 1, 2, and 3 hours after each dose were negative for streptomycin, but hourly urine samples did demonstrate a factor inhibitory to *Bacillus subtilis* that was not found before administration of the drug.

DISCUSSION

The investigations indicate that there is not only a species variation in reaction to streptomycin, but also a considerable variation in individuals of a given species. Thus, it is considered that the mouse is a poor choice of animal for work requiring maintenance of streptomycin blood levels. This is also believed to be true of this animal in respect to other antibiotic work. How the drug is eliminated or destroyed by the mouse at a faster rate than by other animals has not been determined, but examination of the extracts of the liver and spleen indicate that it is not removed by, or stored in, either of these organs. Its appearance in the kidney extracts is attributed to the filtration and concentration procedures of urinary elimination.

For correlation with man, the guinea pig appears to be the more desirable choice of small animal for antibiotic tests. However, it appears probable that

the guinea pig does tend to permit cumulation of the drug to an extent greater than that which has been seen in man. Thus, a dose of 15,000 units per kilogram every three hours was recommended for work requiring maintenance of a blood level of 20 units per cubic centimeter or above in the guinea pig. On trial, the repeated doses were found to provide such a level or above, although single dose administration did not always provide the minimum concentration. Experience to date indicates that in all animals a minimum of three consecutive doses at the proposed therapy interval should be used for obtaining preliminary data on the expected blood level of a drug course.

The rabbit, judging from the very scanty data available, appears to be a satisfactory small animal for studying streptomycin blood levels. It is probable that the blood level is depleted more slowly in the rabbit than in most other animals, and if prolonged intervals are desired between consecutive doses of the drug, the guinea pig or rabbit appears to be the animal of choice.

The experiment with monkeys provided the assay method with its first significant test for day-to-day determination. The infected animals were definitely ill at the beginning of therapy, and, although blood levels did vary from animal to animal and from day to day in the same animal, the data are considered essentially correct. Some of the standard curves were checked at the conclusion of the experiment after the blood had been found free of the drug, and these corresponded very well with the originals. The rate of disappearance of the drug from the blood of each animal was considered additional evidence that the animal variation is considerable. Thus, it is believed that the best streptomycin therapy will result from variation in dose, to maintain a rather narrow blood level range, instead of from the administration of a predetermined dose of the drug.

In man the blood levels seemed to be within the range expected from previous animal work. In the one patient (Patient 4) receiving the dose of drug nearest that administered to the monkey, the blood levels compared closely. The striking findings from human administration were the great concentration of the drug in its passage through the kidney and the prolonged urinary excretion after cessation of therapy. No explanation is advanced for this observation, but certainly it is logical to conclude that a storage of drug is established somewhere in the body. The experience gained so far in attempting to dilute blood and protein solutions containing the drug indicates that streptomycin has a definite affinity for protein, but whether the drug is stored in a protein-rich portion of the body or whether it is only slowly removed from the blood proteins has not been established. Either of these conditions might provide an explanation for prolonged urinary excretion.

Due to the discomfort of continued intramuscular, subcutaneous, or intravenous injection of the drug, and with the hope of eliminating some of the toxicity from the first lots of the drug, oral administration was attempted. Experiments based on penicillin studies were tried, and insignificant absorption of streptomycin from the gastrointestinal tract was demonstrated. To date no satisfactory vehicle for oral administration is available. Due to scarcity of the drug, the work was stopped until more of its chemical and physical character-

istics which can be used as a base for different approaches are known. The consistent finding of the drug in the urine of the dog and man indicates that the problem of oral administration is not insurmountable, and certainly the therapeutic advantage gained would justify further investigation.

Red and white blood counts were followed in some of the rabbits, guinea pigs, monkeys, and human beings during their exposure to streptomycin, and in no case was an abnormal result obtained. Preliminary studies on the partition coefficient of streptomycin in blood indicate that the cells do not carry the drug, since neither water nor saline washing of the centrifuged cells or clot provided an antibiotic material, although both plasma and serum were active. This partition was true whether the streptomycin was added to drawn blood or blood samples were taken from an animal following drug administration.

SUMMARY

1. Streptomycin was administered by subcutaneous, intravenous, intradermal, intramuscular, and intraperitoneal injection to the mouse, guinea pig, rabbit, and man, and blood levels of the drug were determined at various intervals after administration. Maximum blood levels appeared from fifteen to sixty minutes following its injection.

2. For maintenance of blood levels of the drug following one dose, the animal of choice appeared to be, in order, guinea pig, rabbit, monkey, and mouse.

3. Urinary excretion of streptomycin was followed in two patients on an intensive parenteral therapy course of the drug and in one volunteer receiving the drug orally.

4. Administration of streptomycin apparently does not affect the count of red or white blood cells.

5. Therapeutic blood levels of the drug could not be obtained by oral administration in a variety of vehicles.

6. Variation of blood levels from a given dose of streptomycin is seen from animal to animal, and during a therapeutic course, the blood level in the same animal varies from day to day. Similar variation is seen in disappearance of the drug from the blood at the end of the drug course. This pattern is also followed in human beings.

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A STUDY OF THE DIFFUSION OF PENICILLIN ACROSS THE SEROUS MEMBRANES OF JOINT CAVITIES

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MANY infections of joints are caused by bacteria which are sensitive to penicillin. As a prerequisite to the establishment of a plan of therapy for the purulent arthritides, we felt that it was necessary first of all to determine whether there was an exchange of penicillin between the blood and joint fluid. The results of this study are included in the present paper.

PLAN OF STUDY

Penicillin was injected into a knee joint or administered systemically to a group of patients, and assays were performed on joint fluid and serum of each patient to determine the amount of penicillin diffusing across the serous membrane of the joint cavity. Similar studies were done while the patient was under treatment in a fever cabinet to ascertain whether high temperatures would influence the exchange of penicillin between joint fluid and blood. Specimens for penicillin assay on these patients were taken after the patient had been in the cabinet for several hours and the body temperature was 104° F. or over.

The patients studied had a variety of joint diseases: rheumatic fever or rheumatoid, gonococcic, and other pyogenic arthritides. In order to determine whether there was any difference in the diffusion of penicillin into joint fluid from the blood in diseased and normal joints, several patients receiving penicillin, but with no evidence of joint disease, were given up to 100 c.e. of physiologic salt solution into a knee joint, and this fluid was used for penicillin assay.

Several preparations of penicillin were employed: the sodium and calcium salts of penicillin G, the calcium salts of penicillin X, and sodium crystalline penicillin.† Penicillin was given orally in tablets‡ in doses of 100,000 units every two hours; by continuous intravenous infusion in doses of from 100,000 to 200,000 units for periods of from six to twelve hours; and by intermittent intramuscular injection, using from 20,000 to 80,000 units every two hours, from 25,000 to 30,000 units every three hours, and 50,000 units every six hours. When penicillin was injected into a knee joint, up to 200,000 units were dissolved in from 20 to 50 c.e. of physiologic salt solution. Joint fluid for penicillin assay was obtained by aspiration through a spinal needle which had previously been inserted into the knee joint and taped in place for the necessary number of hours. At the desired time the stylet was removed and joint fluid withdrawn. Penicillin

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*Dr. Feffer's contribution to this study was made prior to his entrance into the Armed Forces.

†Supplied by Lederle Laboratories, Inc., Pearl River, N. Y.

‡Supplied by Merck & Company, Rahway, N. J.

TABLE I. SERUM AND KNEE FLUID CONCENTRATIONS OF

PATIENT	JOINT DISEASE	DOSE (UNITS)	CONCENTRATIONS OF							
				5 MIN.	15 MIN.	30 MIN.	1 HR.	2 HR.	3 HR.	4 HR.
B. B.	Pyogenic arthritis	30,000	Serum				.078	.078	.078	.078
			Knee fluid	40						
C. M.	Pyogenic arthritis	30,000	Serum					.039		.039
			Knee fluid	20						
T. K.	Gonococcie arthritis	50,000	Serum				.039	.039	.039	.039
			Knee fluid	40						
C. J.	Gonococcie arthritis	50,000	Serum				.039	.039	.039	.039
			Knee fluid	40						
S. A.	Gonococcie arthritis	50,000	Serum					.078		.156
			Knee fluid					10		5
L. J.	Gonococcie arthritis	50,000	Serum					.039		.156
			Knee fluid					10		5
L. J.*	Gonococcie arthritis	50,000	Serum					.039		.039
			Knee fluid					40		40
Lu. L.	Pyogenic arthritis	50,000	Serum				.078	.312	.312	
			Knee fluid			40	40	40		
J. B.	Pyogenic arthritis	50,000	Serum				.078		.156	
			Knee fluid			20		20		
R. C.	Rheumatic fever	50,000	Serum	0	0	.0195	.039	.156	.312	.156
			Knee fluid	40						
C. M.	Pyogenic arthritis	60,000	Serum	0				.0195		.0195
			Knee fluid	40						
T. K.	Gonococcie arthritis	100,000	Serum				.078	.039	.039	.039
			Knee fluid	40						
A. K.	Rheumatoid arthritis	100,000	Serum				.0195	.0195	.0195	.0195
			Knee fluid	40						
T. K.	Gonococcie arthritis	200,000	Serum				.039	.039	.039	.039
			Knee fluid							
A. K.	Arthralgia	200,000	Serum				0		0	
			Knee fluid					20		
La. L.	Arthralgia	200,000	Serum				0		0	
			Knee fluid					20		

*Fever therapy.

determinations were done on serum and joint fluid at regular intervals by the methods of Rammelkamp¹ and Randall and associates.² No differentiation is made as to the type of penicillin employed because the results were the same with all preparations.

RESULTS

Of the fourteen patients who were given doses of from 30,000 to 200,000 units of penicillin into a knee joint on sixteen occasions, penicillin was found to be present in the knee fluid for from twenty-four to one hundred twenty hours, depending upon the dose (Fig. 1). It was present in the blood on fourteen occasions one hour after intra-articular injection. In one patient, from whom blood was taken at one-half hour after injection into the knee joint, penicillin was present. In each patient the penicillin concentration in the blood was maintained at a nearly constant level throughout the period of observation. The heights of the serum concentrations are shown in Table I. Two patients had a median serum penicillin concentration of .0195 unit per cubic centimeter, five had .039 unit per cubic centimeter, and six had .078 unit per cubic centimeter or better. Although knee fluid concentrations of penicillin of from 10 to 40 units per cubic centimeter were maintained for periods of twelve and twenty-four hours,

PENICILLIN FOLLOWING INTRA-ARTICULAR INJECTION OF PENICILLIN

PENICILLIN (UNITS PER CUBIC CENTIMETER)

5 HR.	6 HR.	7 HR.	8 HR.	9 HR.	10 HR.	11 HR.	12 HR.	18 HR.	24 HR.	48 HR.	72 HR.	96 HR.	108 HR.	120 HR.	144 HR.
.078	.078	.078	.078	.078	.039	.039	.039	0	0	0	0				
							.625								
			.039				.039	.039	0	0					
							.625		.078	0					
.039	.039	.039	.039	.039	.039		.039	0	0	0					
							.156		0	0					
.039	.039	.039	.039	.039	.039	.039	.039	0	0	0					
20							2.5		.312	0					
	.078														
	2.5														
	.312		.156		.078		.039								
5															
	.039														
20															
	.156														
10															
.078	.078	.078					0								
10							5								
			.0195				.0195	.0195	.0195	.0195	0				
									5	.312	.0195				
.039	.039	.039	.039	.039	.039	.039	.039	.039	.039	.039	0	0			
							40		10	1.25	.156	.039			
.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	0			
							20		20	2.5	2.5	0			
.039	.039	.039	.039	.039	.039	.039	.039	.039	.039	.039	.039	.039		.0195	0
							20		20	2.5	1.25	.156		.156	0
0				0			0		0	0					
10							5		2.5	.312	.156	.156	0		
0				0			0		0	0					
10							5		2.5	.625	.312	.078	0		

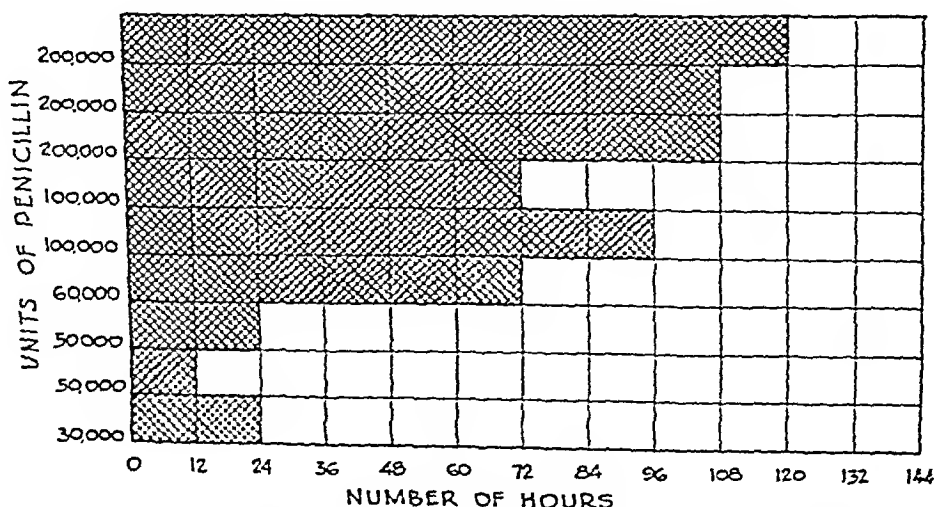


FIG. 1.—Duration of detectable penicillin concentration in knee joint fluid following intra-articular injection.

TABLE II. KNEE FLUID AND SERUM PENICILLIN CONCENTRATIONS

PATIENT	JOINT DISEASE	DOSE (UNITS)	NUMBER OF DOSES	METHOD OF ADMINISTRATION	15 MIN. 30 MIN.	
C. J.	Gonococcic arthritis	20,000 q. 2 hr.	4	Intramuscular	Knee fluid Serum	
B. T.	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	
B. T.*	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	
B. T.	Gonococcic arthritis	20,000 q. 2 hr.	1	Intramuscular	Knee fluid Serum	
G. G.	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	
G. C.*	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	
H. B.	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	.625 2.5
B. J.	Gonococcic arthritis	20,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	0 .625
R. C.	Rheumatic fever	50,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	.0195 1.25
M. J.	Rheumatic fever	50,000 q. 2 hr.	2	Intramuscular	Knee fluid Serum	.0195 .625
C. M.	Gonococcic arthritis	60,000 q. 2 hr.	1	Intramuscular	Knee fluid Serum	
L. P.	Gonococcic arthritis	80,000 q. 2 hr.	6	Intramuscular	Knee fluid Serum	
E. B.	Normal	25,000 q. 3 hr.	2	Intramuscular	Knee fluid Serum	
E. B.*	Normal	25,000 q. 3 hr.	2	Intramuscular	Knee fluid Serum	
E. B.	Normal	25,000 q. 3 hr.	2	Intramuscular	Knee fluid Serum	
R. M.	Gonococcic arthritis	25,000 q. 3 hr.	2	Intramuscular	Knee fluid Serum	.078 1.25
L. C.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
O. W.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
O. W.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
O. W.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
C. A.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
O. J.	Gonococcic arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
M. J.	Rheumatoid arthritis	25,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
L. F.	Gonococcic arthritis	30,000 q. 3 hr.	1	Intramuscular	Knee fluid Serum	
T. K.	Gonococcic arthritis	40,000 q. 4 hr.	3	Intramuscular	Knee fluid Serum	
S. A.	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
S. A.*	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
S. A.	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
L. J.	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
L. J.*	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
L. J.	Gonococcic arthritis	50,000 q. 6 hr.	1	Intramuscular	Knee fluid Serum	
B. S.	Pyogenic arthritis	100,000 continuous- 6 hr.	1	Intravenous	Knee fluid Serum	

*Fever therapy.

FOLLOWING PARENTERAL AND ORAL ADMINISTRATION OF PENICILLIN

CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER)												
1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	6 HR.	7 HR.	8 HR.	9 HR.	10 HR.	11 HR.	12 HR.	13 HR.
.0195	.039	.039	.039	.039	.039	.039	.0195					
.156	.0195	.156	.039	.312	.039	.0195	0					
.0195	.0195	.0195	.0195									
.078	.039	.156	.039									
.039	.078	.078	.078									
.312	.039	.078	.039									
.0195	.0195											
.078	.039											
.039	.039	.039	.039									
.078	0	.078	.078									
.039	.039	0	.039									
.156	.039	.078	0									
.156	.039	.039	.039									
.312	.039	.625	.039									
.312	.078	.078	.078									
.078	0	.156	.039									
.078	.078	.156	.156	.156	.156	.078	.039	.0195	0			
.312	.156	.156	.156		.039		0					
.078	.078	.156	.078	.078	.078	.0195	0					
.312	.156	.625	.078		.039		0					
.039	.039	.039	.039	.039	.039	.039	.039	.0195	.0195			
.625	.156	1.25	.156	1.25	.156	.312	.039	0				
.039	.039	.039	.039	.078	.039	.039	.039	.078	.078	.039	.039	
2.5	.156	1.25	.078	2.5	.156	.625	.078	.625	.078	1.25	.156	
0	.039	.039	.078	.039	.039	.039	.039					
.078	.039	.039	.156	.078	.039	0	0					
.039	.039	.039	.039	.039	.039	.039	.039					
.156	.078	.039	.312	.078	.078	0	0					
.039	.039	.039	.039	.039	.078	.039	0					
.078	.039	0	.156	.039	0	0	0					
.156	.078	.039	.078									
.625	.078	.039	2.5									
.078	.039	0										
.625	.156	.078										
.078	.078	.078										
.156	.078	.039										
.078	.078	.156										
.156	.156	.039										
.078	.156	.156										
.312	.156	.039										
.039	.078	.078										
.312	.156	.039										
.078	.078	.078										
.156	.078	0										
.039	.078	.078										
.156	.039	0										
.078	.078	.156										
.625	.039	0										
.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195
.039	0	0	0	.078	.0195	.0195	0	.078	.0195	0	0	.0195
.039	.039	.039	.039	.039	.039	.078						
.312	1.25	.625	.625	.625	.625	.625	.625					
.039	.625	.625	.312									
	.039	.039	.039	.039	.039	.039						
	.625		.312			.078						
	.039		.039			.039						
	.156		.078			.039						
	.039		.039			.039						
	.312		.078			.039						
	.039		.039			.039						
	.156		.078			.039						
.039	.039	.039	.039	.039	.039	.039	.039					
.156	.312	.312	.312	.312	.312	.156	.0195					

TABLE II—

PATIENT	JOINT DISEASE	DOSE (UNITS)	NUMBER OF DOSES	METHOD OF ADMINISTRATION		
					15 MIN.	30 MIN.
B. S.	Pyogenic arthritis	200,000 continuous 6 hr.	I	Intravenous	Knee fluid	
T. K.	Gonococcic arthritis	200,000 continuous 12 hr.	I	Intravenous	Serum	
A. K.	Rheumatoid arthritis	200,000 continuous 12 hr.	I	Intravenous	Knee fluid	
T. K.	Gonococcic arthritis	200,000 continuous 8 hr.	I	Intravenous	Serum	
J. H.	Gonococcic arthritis	100,000 q. 2 hr.	2	Oral	Knee fluid	
					Serum	

the highest serum concentration observed was .312 unit per cubic centimeter. In no instance was there evidence of absorption of penicillin from the joint fluid into the blood when the concentration in the knee fluid fell below .156 unit per cubic centimeter. Two patients with arthralgias secondary to a systemic disease showed no evidence of absorption of penicillin from the knee joint into the blood following an intra-articular injection of 200,000 units.

Of a group of twenty-two patients who were given penicillin orally or parenterally on thirty-seven occasions, all had significant concentrations in their sera. Penicillin was found to have diffused into the joint fluid in all patients except the one who was given the drug orally (Table II). Several patients had no joint disease, and the injected physiologic salt solution was used as joint fluid. As in the case of intra-articular injection, the absorption of parenterally administered penicillin into the joint was almost constant, with median concentrations of penicillin of .0195 unit per cubic centimeter of joint fluid in six instances, .039 unit per cubic centimeter in fifteen, and .078 unit per cubic centimeter or better in fifteen. The amount of penicillin which diffused into the joint fluid was usually independent of the serum concentration. Joint fluid concentrations of penicillin varied up to 50 per cent of the highest serum concentration. Occasionally the joint fluid concentration was higher than the serum concentration when the patient was receiving intermittent intramuscular injections, demonstrating that the drug apparently was excreted more slowly from the joint fluid than from the blood. We feel that these results represented an adequate sampling, as blood and joint fluid for penicillin assay were taken at one- or two-hour intervals for from six to twelve hours in twenty studies, for four hours in seven, and for two or three hours in ten.

In about one-half of the studies the blood and joint fluid were taken directly following the start of penicillin therapy, and in the other one-half the studies were performed after the patient had been under treatment for one or more days. The results were the same regardless of the duration of therapy indicating that penicillin did not accumulate in joint fluid.

Penicillin was found to diffuse rapidly into the joint fluid when given systemically. Of the three patients from whom joint fluid was obtained fifteen minutes after the first parenteral injection, penicillin was present in the joint fluid of two. In the other patients, penicillin was found in the first specimen, which was obtained one-half and one hour after the drug was started.

CONT'D

CONCENTRATIONS OF PENICILLIN (UNITS PER CUBIC CENTIMETER)												
1 HR.	2 HR.	3 HR.	4 HR.	5 HR.	6 HR.	7 HR.	8 HR.	9 HR.	10 HR.	11 HR.	12 HR.	13 HR.
.0195	.0195	.0195	.0195	.0195	.0195	.0195	.0195					
.312	.625	.625	.625	.625	.625	.039	.0195					
.0195	.0195	.0195	.0195	.0195	.0195	.039	.039	.0195	.039	.039	.039	.039
.078	.039	.0195	.0195	.0195	.0195	.312	.078	.0195	.078	.039	.039	0
.0195	.0195	.0195	.0195	.0195	.0195	.039	.039	.0195	.039	.039	.039	.0195
.156	.156	.156	.156	.156	.156	.156	.156	.312	.312	.312	.312	.0195
.039	.039	.039	.039	.039	.039	.039	.039	.039	.039			
1.25	2.5	2.5	5	5	1.25	1.25	.312	.0195	0			
0	0	0	0									
.078	0	.078	0									

In several patients determinations were made on blood and serum for several hours after the drug was discontinued, and the joint fluid was found to contain penicillin for from one to three hours longer than the blood, corroborating the observation that penicillin was excreted more slowly from the joint fluid than from the blood.

The effects of fever therapy in the exchange of penicillin between joint fluid and blood were studied in six patients. A comparison was made in each patient of the amount of penicillin transferring between the blood and joint fluid before, during, and after fever therapy. One patient received the penicillin intra-articularly and five were given the drug intramuscularly. In two of the latter group absorption into the knee fluid was found to have occurred in slightly higher concentrations during fever therapy than under normal conditions. However, fever therapy did not influence the amount of penicillin transferred across the serous membranes in the remaining four patients.

Penicillin has been found to be irritating to serous membranes, causing a pleocytosis on injection into the spinal fluid³ or pleural space.⁴ We found that such a reaction occurred when penicillin was injected into a joint. There was an increase in the number of white blood cells over a forty-eight hour period which receded to normal over the next few days.

DISCUSSION

Rammelkamp and Keefer⁵ found serum penicillin concentrations up to .007 unit per cubic centimeter following an injection of 10,000 units of the antibiotic into a knee joint or bursa. Florey and Heatley⁶ demonstrated absorption of penicillin into the blood for from seven to twenty-four hours following an intra-articular injection of from 100,000 to 120,000 units of the drug. However, they did not report any serum concentrations. Herrell and associates⁷ noted that up to 50 per cent of the serum concentrations of penicillin will diffuse into joint fluid with parenteral administration. Ory and co-workers⁸ were able to detect penicillin concentrations of .03 and .06 unit per cubic centimeter of joint fluid from one-half to two and one-half hours following intramuscular injection of from 15,000 to 20,000 units. Balboni and associates⁹ reported consistently significant levels of penicillin in the joint fluid of seven patients following the intramuscular injection of from 25,000 to 40,000 units of penicillin.

The joint fluid levels reported by these investigators are higher than those obtained by Ory and co-workers and by us with comparative doses.

Although we were able to demonstrate the transfer of penicillin between blood and joint fluid, analysis of our results shows that when penicillin was given systemically, joint fluid concentrations of .0195 unit per cubic centimeter were found in 16 per cent of the studies, .039 unit per cubic centimeter in 41 per cent, and .078 unit per cubic centimeter or better in 41 per cent. The highest joint concentration of penicillin obtained was .625 unit per cubic centimeter in two patients on one each of a series of determinations. When the penicillin was injected into a knee joint, absorption into the blood reached a level of .0195 unit per cubic centimeter in 15 per cent of the patients, .039 unit per cubic centimeter in 39 per cent, and .078 unit per cubic centimeter in 46 per cent. The greatest amount of penicillin absorbed into the blood from the joint space was .312 unit per cubic centimeter.

When the joint fluid concentrations of penicillin obtained with systemic administration of the drug are correlated with the concentration usually needed to kill the various penicillin-sensitive bacteria, particularly staphylococci, it is found that in a significant percentage of instances insufficient amounts of penicillin diffuse into the joint fluid to control the infection of the joint. Similarly, insufficient amounts of penicillin are absorbed into the blood from a joint space to care for other sites of infection. These observations are confirmed by our clinical experiences with patients who had pyogenic arthritis and periarticular inflammation and/or bacteremia in whom penicillin had to be given systemically as well as intra-articularly to control the extra-articular infection. We have also found systemic penicillin alone to be inadequate in the treatment of pyogenic arthritis complicating a bacteremia.

Correlation of various factors such as the height of serum concentrations of penicillin, duration of treatment, method of administration, and type of joint disease, with the amount of penicillin diffusing across the serous membranes of joints, reveals that although there is a tendency toward greater diffusion with high serum or joint fluid concentrations, there is no constant relationship between the two. Neither the duration of treatment nor the method of administration influenced the transfer of penicillin across the serous membrane. Except in two patients with arthralgia, who had no absorption of penicillin from the joint into the blood, we found the diffusion across the serous membrane to be the same irrespective of the presence or type of joint disease. Fever therapy did not significantly alter the diffusion of penicillin, for in only two of six patients was there a slight increase in the amount of penicillin exchanged between joint fluid and blood.

SUMMARY AND CONCLUSIONS

1. Penicillin will diffuse across the serous membranes of joints when given systemically or intra-articularly.

2. Low concentration of penicillin in the joint fluid following the systemic administration and in the blood following intra-articular injection were obtained in a significant number of studies.

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OUTBREAK OF HEMOLYTIC STREPTOCOCCUS THROAT INFECTION CONTROLLED BY SULFADIAZINE

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THE prophylactic use of sulfonamides against bacterial infections has assumed considerable importance during recent years. Sulfanilamide has been used in preventing streptococcal upper respiratory infections and relapses in rheumatic fever.¹⁻³ Kuhns and associates⁴ found that sulfadiazine in amounts as low as 2 Gm. daily diminished the meningococcal carrier rate as well as the incidence of meningococcal meningitis. Rubenstein and Foley⁵ studied two outbreaks of scarlet fever, one occurring in an orphanage due to type 1 streptococcus and the other occurring in a schoolroom due to a type 5 streptococcus. In both epidemics small daily doses of sulfonamides reduced to a minimum the number of persons carrying the incriminated organism.

During the 1944-1945 holiday season an epidemic of sore throats occurred among the staff of a United States Army General Hospital on Oahu, Territory of Hawaii. Due to the burden of casualties from the Philippine Campaign at this time, it was necessary to take quick and effective action in order to maintain operation of the hospital. This was carried out successfully with therapeutic and prophylactic use of sulfadiazine.

The first patient was admitted Dec. 28, 1944. Two were admitted Dec. 31, 1944, and three more, Jan. 1, 1945. On Jan. 2, 1945, eight more of the hospital personnel were admitted at various times of the day. The earlier individuals showed the presence of large numbers of beta hemolytic streptococci in throat cultures. It was apparent that a fairly large epidemic of streptococcal sore throat was going to disable the staff of the hospital, unless the proper action were taken immediately. The protection of the hospital patients was also of prime importance. In order to control the situation quickly the following measures were taken: (1) detection of all cases and suspected cases by screening of the personnel; (2) isolation of all cases and suspects; (3) efforts to determine the origin of all known cases; and (4) administration of sulfadiazine prophylactically to personnel of the staff.

These measures were accomplished in the following manner. A team of medical officers examined all enlisted personnel, by roster, as they passed through the mess line. All men were questioned as to how they felt; their noses and throats were examined; and their temperatures were determined when indicated. All officers and nurses were examined after lunch at the outpatient clinic. Through this process twenty-one new patients, of whom two were nurses, were admitted to the hospital and placed in contagious wards.

The epidemiology of the outbreak was investigated. To aid in this study, a map of the hospital area was prepared, and all cases were marked on the map as to the location of the patient's work and his sleeping quarters. Throat cultures were performed on all mess personnel to detect the presence of carriers. Histories on each case were carefully checked for a common source. The evidence gathered by these methods of approach all pointed to a probable origin at a dance held by the enlisted men of the organization Dec. 27, 1944. Of the WAC personnel who attended the dance, two were admitted with the disease.

Prophylactic administration of sulfadiazine was started Jan. 3, 1945, and continued for five days by giving the enlisted personnel a tablet (7½ gr. of sulfadiazine) before each meal under the supervision of the noncommissioned officers. Tablets were made available to the officers and nurses at the mess hall, and they were instructed to take one tablet three times daily. Following the administration of the drug, a marked decrease in the incidence of sore throats was noted. Between Dec. 28, 1944, and Jan. 3, 1945, there was a total of thirty-five patients admitted with hemolytic streptococcus sore throats. Following the administration of sulfadiazine prophylaxis, which started Jan. 3, 1945, there were only seven cases up to Jan. 15, 1945, when the last case occurred. No reactions due to sensitivity to the sulfadiazine were noted.

TREATMENT

Patients with the more severe cases were given an initial dose of 4 Gm. of sulfadiazine followed by 1 Gm. every four hours. This was maintained for periods varying from two to eight days. Response to therapy was excellent. Some of the patients admitted were not very ill and were treated symptomatically. Those with mild cases responded well, even without the use of sulfonamides. Of the entire group there was only one patient who was seriously ill. He had a profuse pharyngitis with considerable edema of the fauces and bilateral cervical adenopathy. On sulfadiazine, his temperature dropped from 103° F. to normal in seventy-two hours, and he made an uneventful recovery.

CLINICAL LABORATORY FINDINGS

Throat cultures were made daily from individuals suspected of hemolytic streptococcus infection. Culture media in each case were a blood agar plate and Löffler's serum medium. The throats at time of culture in the positive cases were uniformly inflamed. Pure cultures of hemolytic streptococci were usually found in these individuals. In one moderately severe case a mixture of *Staphylococcus aureus* and *Streptococcus pyogenes* was found initially. After a few days' treatment with sulfadiazine the hemolytic streptococci began to disappear, and indifferent and green-producing streptococci, micrococci, and *Neisseria* appeared in the cultures. During convalescence, positive cultures would frequently recur after several negative daily cultures. Two patients had clinical relapses.

The leucocyte count on admission varied over a broad range from 5,000 to 20,000. In general, the leucocyte count did not correlate very closely with the clinical severity. Urinary findings were essentially normal.

CULTURAL AND SEROLOGIC STUDIES

Twenty-six strains of hemolytic streptococci isolated from patients in this outbreak were studied in detail. Lancefield precipitin methods were employed for serologic analysis.

Methods and Materials.—Cultures were isolated on horse blood agar plates. Hemolysis, if its type were questionable, was checked in deep agar plates. One strain showed a minute zone of beta hemolysis and proved to belong to Group H (Table I).

TABLE I. DISTRIBUTION OF STREPTOCOCCUS GROUPS AND TYPES IN TWENTY-SIX STRAINS OF HEMOLYTIC STREPTOCOCCI STUDIED

GROUP*	NUMBER	PER CENT	TYPE†	NUMBER	PER CENT
A	25	96	3	1	4
H	1	4	36	18	72
			38	2	8
			*NT‡	4	16
Total				25	100

*Groups of sera employed: A, B, C, D, E, F, G, H, K, L.

†Types of sera employed: 1, 3, 5, 6, 10-12, 14, 17, 18, 19, 24, 26, 28, 30, 31, 36, 37, 38, 42, 43.

‡No type obtained in any serum.

Extracts for grouping and typing were prepared from a single broth culture. Forty cubic centimeters of tryptose phosphate broth at pH 7.6 to 7.8 were heavily inoculated with a young culture of streptococcus. Care was taken that incubator temperature did not rise over 37° C. during growing of the broth culture, since higher temperatures might prevent formation of M-substance.⁶ At eighteen hours' incubation, the culture was centrifuged, and the packed cells were resuspended in 0.6 c.c. of N/20 HCl. The suspension was mixed, heated in boiling water for ten minutes with shaking, cooled quickly, and one drop of phenol red indicator solution added. One drop of N/5 NaOH was added to reduce final volume of extract; then neutralization was completed to a pink color with N/20 NaOH made up in M/60 phosphate buffer at pH 7.0 (0.25 Gm. Na₂ HPO₄ in 100 c.c. N/20 NaOH). A final pH of 7.0 is desired. The mixture was centrifuged at 2,500 r.p.m. for forty-five minutes and the clear supernatant fluid removed with a capillary pipette. It is essential that the supernatant be entirely clear.

The extract may be used for both grouping and typing. In our hands, Lancefield's original procedure⁷ for grouping extract in which 0.1 per cent glucose broth was used as culture medium gave a better extract for grouping in that larger, more distinct precipitates were formed. It is suggested that this modification be tried in the event of any difficulty encountered in grouping reactions. In carrying out serologic tests on streptococci, reactions should be set up the same day that the extract is prepared. If this is not practicable, prepare the extract with sterile technique. Even slight degrees of bacterial contamination may produce negative or inconclusive results in grouping and typing.

Grouping and typing reactions when carried out in capillary tubes as described by Swift, Wilson, and Lancefield⁸ result in a great economy in amount

of serum used. Care must be taken that serum is quite clear, for slight amounts of precipitate may simulate a positive reaction. A small rubber bulb for drawing serum into capillaries is recommended, since the viscosity of the serum makes filling by capillary action slow. A convenient mount for capillary tubes consists of a 150 mm. Petri dish filled one-quarter inch deep with a mixture of one part paraffin and one part petrolatum.

Inconclusive results should be checked by a microprecipitin procedure, using tubes 0.3 mm. in diameter drawn from 6 mm. tubing. Dilutions (1:2, 1:4, 1:8, 1:16) of serum against undiluted extract, and dilutions of extract against undiluted serum, may be necessary to demonstrate specific type in presence of a prozone reaction.

Sera available for grouping and typing in this study are shown in Table I.

Mouse passage was employed to enhance M-substance in fourteen cases. Storage of cultures for four months prior to acquisition of typing serum possibly affected the ability of these strains to elaborate M-substance. Four passages were made, using intraperitoneal injection of 0.05 c.c. of a broth culture and recovering the strain when the mouse was moribund. The peritoneal washings were injected directly into the next mouse. Table III illustrates results of this technique in increasing positive results of typing.

Since sulfadiazine was employed in checking the epidemic, sulfadiazine sensitivity *in vitro* was determined. To test sulfadiazine sensitivity, strains were grown in clear tryptose semisolid broth containing 0.1 per cent glucose and graded amounts of sulfadiazine. Table II shows results. All strains of Group A were inhibited by a concentration of 0.2 mg. per 100 c.c. of sulfadiazine. The Group H strain was more resistant to sulfadiazine and grew in concentration of 1.0 mg. per 100 c.c. of broth.

TABLE II. SULFADIAZINE SUSCEPTIBILITY OF STREPTOCOCCI STRAINS

NUMBER OF STRAINS	SULFADIAZINE CONCENTRATIONS IN CULTURE MEDIUM IN MG. PER 100 C.C.							
	0.05	0.1	0.15	0.2	0.3	0.5	1.0	CONTROL
1	++	-	-	-	-	-	-	+++
5	+++	++	-	-	-	-	-	+++
18	+++	++	+	-	-	-	-	+++
2	+++	+++	++	-	-	-	-	+++
1	+++	+++	+++	+++	+++	++	+	+++
(Group H) Control (C203MV)	+++	++	+	-	-	-	-	+++

+, Barely perceptible clouding of medium at twenty-four hours.

++, Definite clouding of medium at twenty-four hours.

+++ Heavy growth at twenty-four hours.

Observations.—

Morphology: Three colony types were observed: smooth, mucoid, and rough. Smooth colonies were slightly raised, with indistinct margins and a colony diameter of from 0.5 to 0.7 mm. at twenty-four hours. Mucoid colonies were larger than the smooth colonies, with less distinct margins, a tendency to spread, and a slightly sticky consistency. Rough colonies had undulant margins, a raised dense center, and glistening surface and remained intact when touched with a wire. Smooth and mucoid colonies were evidently matt variants. The

rough colonies tended toward glossy variants, but they still retained matt qualities.

Serologic Analysis.—Cultures were grouped at the time of the epidemic but could not be typed until four months later when serum became available. Storage presumably increased the difficulty of obtaining adequate typing reactions due to the loss of M-substance.⁹ Table I shows group and type distribution. Twenty-five Group A strains and one Group H were identified. Of the Group A strains, eighteen were type 36, two were type 38, and one was type 3. Four strains which could not be typed failed to kill mice in a dose of 0.1 c.c. of an eighteen-hour broth culture intraperitoneally after four passages. It seems probable that these strains lost virulence and M-substance due to prolonged cultivation.

TABLE III. COLONY MORPHOLOGY, TYPE, AND VIRULENCE RELATIONSHIPS IN GROUP A STRAINS

COLONY MORPHOLOGY	TYPE				DIRECT TYPING	TYPING AFTER MOUSE PASSAGE (VIRULENT†)	NEGATIVE FOR TYPING AFTER MOUSE PASSAGE (NONVIRULENT‡)
	3	36	38	NT*			
Smooth		8(32%)			7(28%)	1(4%)	0
Mucoid	1(4%)	1(4%)	1(4%)		0	3(12%)	0
Rough		9(36%)	1(4%)	4(16%)	3(12%)	7(28%)	4(16%)
Total	1(4%)	18(72%)	2(8%)	4(16%)	10(40%)	11(44%)	4(16%)

*No type obtained.

†Mouse killed in twenty-four hours by 0.1 c.c. eighteen-hour broth culture intraperitoneally.

‡Mouse not killed in twenty-four hours by 0.1 c.c. eighteen-hour broth culture intraperitoneally.

Table III shows the relation of colony types and demonstrability of M-substance. Of eight smooth colony strains, seven typed directly and one required mouse passage. Of fourteen rough colony strains, three typed directly. Of the remaining eleven, seven typed after mouse passage. The remaining four strains failed to type at all. Mucoid colony strains required mouse passage before they could be typed. Strains which regained M-substance on mouse passage also showed definite mouse virulence.⁸ Increase in virulence to at least ten times the original level was acquired by mouse passage.

DISCUSSION

A variety of types has been found in studies in streptococcal disease, including scarlet fever and rheumatic fever, as well as in carrier surveys.¹⁰ In this outbreak the predominant organism isolated was the type 36 hemolytic streptococcus. This organism proved to be inhibited in culture by relatively small amounts of sulfadiazine. The clinical outbreak was quickly brought under control with the use of sulfadiazine (1½ Gm. per day to all contacts) combined with an intensive search for, and isolation of, all clinical cases. In addition, the more severe clinical cases responded remarkably well to therapeutic doses of this drug. It is recognized that strains of the same species of organism may vary widely in their susceptibility to sulfonamides. Strains may become resistant to these drugs upon cultivation in media containing minute amounts of sulfonamides, or such strains may be isolated from individuals who have been

treated with amounts of the drug insufficient to bring about the elimination of the parasite from the host. It is reasonable, therefore, to assume that outbreaks of sore throat will vary in their response to the prophylactic administration of sulfadiazine. However, the use of this drug prophylactically in circumscribed outbreaks of streptococcal disease is well justified. Bacteriologically, the types demonstrated lead to the conclusion that type 36 was the predominant causative strain in this outbreak. The presence of additional types, and even of additional groups, of streptococci in an acute outbreak must be taken into consideration. One case exhibited a heavy concentration of Group H streptococcus on initial throat culture. This strain was moderately sulfonamide sensitive and did not kill mice in a dose of 0.1 c.c. of broth culture intraperitoneally. Epidemic outbreaks may be either mixed types or predominantly a single type. Although it is impossible to state categorically that the type 38 or type 3 strain might not have increased in incidence during this epidemic, it is apparent that, at the time the outbreak was checked, the principal inciting agent was *Str. pyogenes*, Group A, type 36.

CONCLUSIONS

An outbreak of streptococcus pharyngitis demonstrated to be due to Group A hemolytic streptococci was quickly brought under control with sulfadiazine prophylaxis combined with other control measures.

Eighteen type 36, two type 28, one type 3, and four nontypable strains were isolated from the outbreak. Responsible type was most probably the type 36 hemolytic streptococcus.

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THE SEROLOGIC DIAGNOSIS OF ENDEMIC TYPHUS

III. THE INCIDENCE AND TITER OF COMPLEMENT-FIXING ANTIBODIES IN RANDOM SAMPLES OF THE POPULATION IN ENDEMIC AND NONENDEMIC TYPHUS AREAS

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IT HAS been recognized for at least a decade that complement-fixing antibodies were demonstrable in the serum of patients who recovered from typhus. Castaneda¹ showed this to be true at least six years after acute infection, and Bengtson and Topping² have shown that they persist in most instances for at least several years. In our own experience, it has been found that the reaction occurs in a relatively high titer for variable periods of time.³

The specificity of the complement fixation test in endemic typhus, using a rickettsial antigen, was demonstrated by Bengtson and Topping,² and the test has been adopted in numerous public health laboratories as a routine diagnostic procedure. In our hands, this has been true for the past two years. Interpretation of the results has been difficult, however, because of the lack of knowledge as to what really constituted a diagnostic reaction and, furthermore, the lack of any information as to the incidence of reactors in the general population. The purpose of this report is to shed some light upon the latter question.

SOURCE OF BLOOD SPECIMENS

In this study, some 900 sera from bloods collected in the course of a routine serologic survey for syphilis, and representing a random sample of the population, were examined. Five hundred of the specimens were from southern Alabama, Houston County, and represent an area in which typhus is endemic. One-half of these bloods were from persons giving an urban address and one-half from persons with a rural address. Four hundred of the specimens came from residents of northern Alabama, Morgan County, where typhus is of rare occurrence. The residence, whether urban or rural, of these individuals was unknown.

RESULTS

In summary, the results of complement fixation tests run on these 900 specimens are presented in Table I.

With regard to the distribution of the fifty-eight reactors found in the specimens from Houston County, twenty-three gave an urban and thirty-five a rural residence.

The end titer of a reacting serum was considered to be that dilution in which complement fixation was 3 plus or stronger. On this basis, an analysis of the fifty-nine reacting sera is presented in Table II.

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TABLE I. RESULTS OF COMPLEMENT FIXATION TESTS RUN ON RANDOM SAMPLES OF POPULATION IN ENDEMIC AND NONENDEMIC TYPHUS AREAS

SOURCE OF SPECIMENS	NUMBER OF SPECIMENS EXAMINED	NUMBER OF SPECIMENS GIVING A COMPLEMENT FIXATION REACTION OF AT LEAST 3 PLUS IN A DILUTION OF 1:4 OR HIGHER
Morgan County (nonendemic area)	400	1
Houston County (endemic area)	500	58

TABLE II. NUMBER OF SPECIMENS GIVING 3 PLUS OR STRONGER REACTION IN A 1:4 OR HIGHER DILUTION OF SERUM

DILUTION OF SERUM	1 TO 4	1 TO 8	1 TO 16	1 TO 32	1 TO 64	1 TO 128	1 TO 256	TOTAL
Number of sera reacting	16	12	13	7	5	4	2	59
Per cent of sera reacting	26.9	20.3	22	11.8	8.4	6.7	3.5	

CONCLUSIONS

1. In a section of the United States where typhus is endemic, an examination of sera for complement-fixing antibodies from a random sample of the population shows the incidence of reactors to be high.
2. In a nonendemic area, the incidence of reactors is almost nil.
3. The titer of complement-fixing antibodies detected in presumably normal individuals varied from 1:4 to 1:256.
4. The significance to be attached to a positive complement fixation test for typhus in a febrile patient living in an area of endemic typhus must be weighted by the foregoing observations.

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INTERPRETATION OF FINDINGS IN THE CEREBROSPINAL FLUID*

II. THE TECHNIQUE AND SYSTEMATIC INTERPRETATION OF THE ALBUMIN-GLOBULIN RATIO IN CEREBROSPINAL FLUIDS

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NUMEROUS methods have been devised for determining the albumin-globulin ratio in cerebrospinal fluids,¹⁻¹¹ and considerable importance has been attached to their diagnostic significance. All of the current methods are based on the assumption that the globulin present in cerebrospinal fluid precipitates, as in blood, by 50 per cent saturation with ammonium sulfate. Data presented in this paper indicate that this assumption is true only when considerably higher concentrations of protein are present than in normal cerebrospinal fluids. Some authors believe that even a crude qualitative determination of the ratio might replace the gold reaction. While this was refuted in an earlier paper¹² on the basis of experimental work, it seemed wise to undertake an objective evaluation of the matter. To do this it was necessary to re-examine technical procedures and to establish the general principle of a systematic interpretation by complete syndromes.

EXPERIMENTAL STUDY

The current methods of determining the albumin-globulin ratio in cerebrospinal fluids¹⁻¹¹ uniformly use 50 per cent ammonium sulfate as if it were a specific globulin reagent. Actually, it provides a crude separation of albumins and globulins only under very restricted conditions. The following simple experiment may best serve to correct the above misconception.

A mixture of 250 mg. of globulin and 500 mg. of albumin per 100 c.c. was prepared. Its albumin-globulin ratio was 2:1; the protein content, 750 mg. per 100 c.c. The globulin and albumin were prepared from presumably normal human serum, diluted 1:10, by repeated precipitations with 50 and 100 per cent ammonium sulfate, respectively, followed by dialysis and protein determination by micro-Kjeldahl. Various dilutions were prepared with physiologic saline, from 750 mg. per 100 c.c., which corresponds to a serum dilution 1:10, to 15 mg. per 100 c.c., which corresponds to the lowest protein concentration encountered in cerebrospinal fluids. After 50 per cent saturation with ammonium sulfate, followed by centrifuging, the globulin value was determined turbidimetrically. The higher the dilution, the greater the deviation of the albumin-globulin ratio from the known value (2:1); the most marked deviations occurred with protein contents ranging from 15 mg. to about 150 mg. per 100

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*The first paper of this series, stressing the necessity of interpreting every cerebrospinal fluid finding as an integral part of complete syndromes, is number 16 of the references.

e.e. This source of error may lead to incorrect results in routine examinations, since the protein concentration of specimens submitted for study frequently falls in the range mentioned (see Fig. 1).

Theoretically, these results may not be new, but they emphasize the inherent danger in current methods.

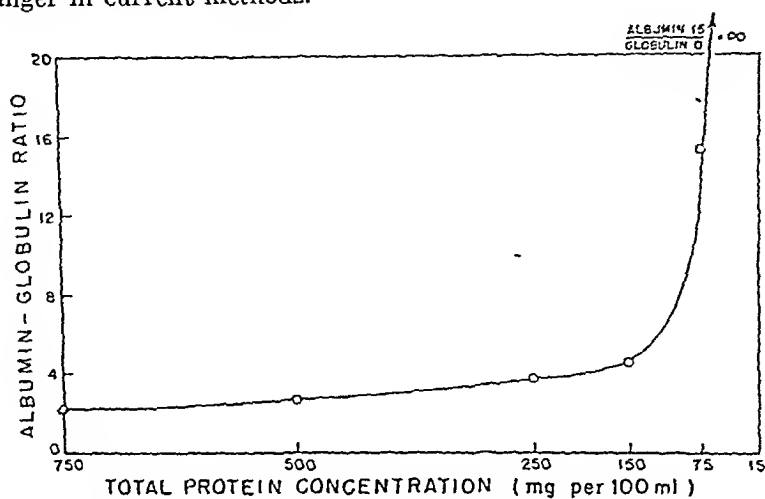


Fig. 1.—Dilution error using 50 per cent ammonium sulfate.

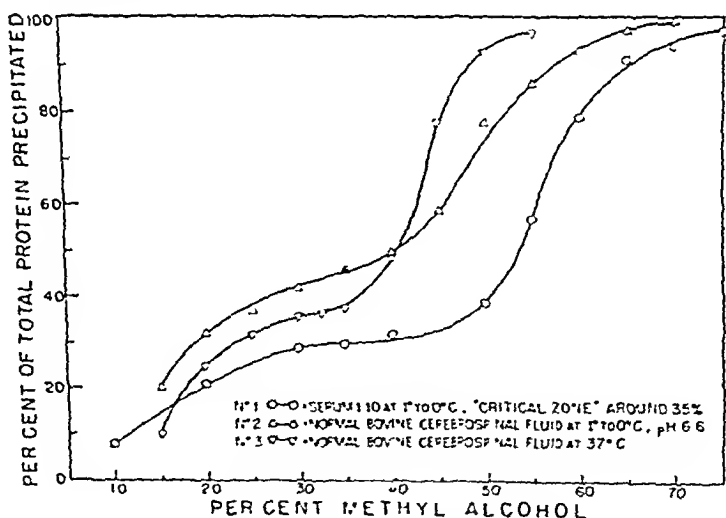


Fig. 2.—Fractionation curves with methyl alcohol.

Numerous fractionation curves were determined with serum in higher dilutions. Various salting-out procedures were tried, both in sera and cerebrospinal fluid, and one observation in technique was made that seemed important under the particular conditions of low protein content in cerebrospinal fluids. In salting-out procedures, the high specific gravity of the mixtures prevents collection of the minute amounts of precipitate even when an angle centrifuge¹² is used. When methyl alcohol¹⁴ was substituted for ammonium sulfate, however,

the globulin precipitate was easily collected with an ordinary centrifuge. Thus, it was possible to establish fractionation curves equivalent to those of normal cerebrospinal fluid, while current methods either fail entirely or yield values of questionable significance (see Figs. 1 and 3).

Liu and Wu¹⁴ prefer precipitation at low temperature to avoid denaturation. Denaturation at 37° C., however, proved advantageous in determining the albumin-globulin ratio. Moreover, at low temperature no critical zone was observed, but at 37° C., such a zone, even if indistinct, seemed to lie around 33 per cent. The gold reaction was also used as a check on the optimal separation of globulins and albumins. On the basis of these findings, the use of 33 per cent methyl alcohol at pH 6.6 and 37° C. was adopted.

Fractionation curves of normal human cerebrospinal fluid could not be established because of the large amount (2,000 c.c.) of material required. Bovine cerebrospinal fluid was therefore substituted, since it has been found to have the same protein content and to give the same gold curve as normal human cerebrospinal fluid.¹⁵ Before pooling the bovine cerebrospinal fluids, total protein determinations and colloidal gold tests were made to be sure that only normal fluids were included.

The following study of the albumin-globulin ratio of human cerebrospinal fluid was undertaken. The total protein was determined by micro-Kjeldahl, the percentage of error being from 3 to 5 per cent. From 0.4 to 0.75 mg. of protein was required for reliable results. Cerebrospinal fluids of high protein concentration were diluted to approximately 75 mg. per 100 c.c. or less. The

TABLE I. ALBUMIN-GLOBULIN RATIO IN CEREbroSPINAL FLUID USING METHYL ALCOHOL

SERIAL NUM- BER	CEREbroSPINAL FLUID	TOTAL PROTEIN (MG. PER 100 C.C.)		ALBUMIN-GLOBULIN RATIO			
		CEREbro- SPINAL FLUID USED FOR MICRO- KJELDAHL (C.C.)	TOTAL PROTEIN (MG. PER 100 C.C.)	CEREbro- SPINAL FLUID USED FOR FRACTIONA- TION (C.C.)			RATIO
					GLOBULIN (MG. PER 100 C.C.)	ALBUMIN (MG. PER 100 C.C.)	
1}	Normal	2	{25	4	{10	{17	2
2}		2	{23		{ 7	{14	
3	Known syphilitic	2	{21		{ 6	{17	2.6
4	Asymptomatic	2	{20	4	{ 8	{20	
5	neurosyphilis	1	60	3	11	26	2.4
6	Paretic formula	0.5		4	13	50	4
7	Acute neuro-		132	2	31	97	3
8	syphilis	1	130	½*	28	99	
9	(Herxheimer's		85	3	7	81	11
10	reaction)			½			
11	Tuberculous	0.5	237	3	13	219	17
12	meningitis			½			
13	Subarachnoid	0.5	348	5	11	343	31
14	block			½			

*The fractions indicate the dilution of the cerebrospinal fluid that had to be used to reduce its protein content to 75 mg. per 100 c.c. or less.

pH, which becomes markedly alkaline after the fluids have stood, was adjusted by adding one-tenth volume of phosphate buffer of pH 6.6 and about $M/7$ concentration. One volume of methyl alcohol was added to two volumes of buffered cerebrospinal fluid, and the mixture was kept at 37° C. overnight in tightly stoppered centrifuge tubes with pointed bottoms. The amounts of cerebrospinal fluid sufficient for an accurate determination (see Table I) were estimated on the basis of the total protein, the gross appearance, the number of cells, the complement fixation test, and the gold reaction (see Table II). The fractions were separated in ordinary centrifuges, and the values of both globulins and albumins were directly determined, when possible, in duplicate. In general, the protein values checked surprisingly well. After centrifuging, the supernatant was removed, the sediment was washed once with 33 per cent methyl alcohol and then added to the supernatant, and the mixture was precipitated with trichloroacetic acid. To obtain sufficient material within the normal range, a pool of previously tested cerebrospinal fluids was used. In Table I is shown a series of representative cases studied.

The technique described is, of course, inadequate for routine examinations, but it provided a satisfactory standard for the present investigation.

SYSTEMATIC INTERPRETATION OF THE ALBUMIN-GLOBULIN RATIO

The systematic interpretation¹⁶ of any cerebrospinal fluid finding requires its evaluation as an integral part of a complete syndrome (see Table II). Moreover, a correct interpretation is inconceivable without a satisfactorily standardized technique, which would be difficult to adapt to a routine determination of the albumin-globulin ratio in cerebrospinal fluid.

The current way of interpreting the albumin-globulin ratio may best be illustrated by Fig. 3, copied from Kafka.⁴ The globulin values shown by Kafka are relative and not directly comparable with the total protein values; both were obtained by a rather inaccurate volumetric determination, by centrifuging. In the range up to 150 mg. per 100 c.c., the total protein values were calculated in milligrams per 100 c.c. The impression is given in this schematic presentation that the albumin-globulin ratio differentiates, besides the normal, not only syphilis and nonsyphilis, but even three forms of neurosyphilis.

The differences in the albumin-globulin ratio obtained by Kafka⁴ in the range from 20 to 150 mg. per 100 c.c. of total protein nearly paralleled the curve obtained when graded dilutions of a mixture of known values were tested (see Fig. 1). Accordingly, there remains only the technically more reliable differentiation between the groups clinically interpreted as "progressive paralysis" and "nonsyphilitic meningitis."

High albumin-globulin ratios are not characteristic of "nonsyphilitic meningitis," such as tuberculous meningitis (see Table I). They are encountered also in acute syphilitic meningitis and in block in the absence of meningitis. Consequently, the problem is to determine the common pathogenetic factor in this etiologically heterogeneous group. To do this, the albumin-globulin ratio must be correlated with the complete syndrome (see Table II). The albumin-globulin ratio invariably is high when fibrin is present. Fibrin is the simplest indicator of a marked increase in the passage of blood proteins into the cerebrospinal fluid.

TABLE II. SYNDROMES OF FIVE GENERALLY OBLIGATORY EXAMINATIONS OF CEREBROSPINAL FLUID (TESTS MADE ON SAME FLUIDS AS IN TABLE I.)

SERIAL NUM- BER	(1) GROSS APPEAR- ANCE	(2) CELLS PER C.MM.		(3) TOTAL PROTEIN (MG. PER 100 C.C.)	(4) COMPLE- MENT FIXATION TEST FOR SYPHILIS		(5) QUANTITATIVE GOLD REACTION ^{1,2*}												EVALUATION OF SYNDROMES		
					BLOOD	CEREBRO- SPINAL FLUID	SERIAL DILUTIONS												TYPE OF GOLD REACTION	PERME- ABILITY AS INDICATED BY SYNDROME	ALBUMIN- GLOBULIN RATIO AS INDICATED BY SYN- DROME
							1:15	23	34	51	76	114	171	256	384	576					
1	Normal	0/3	0	25	0	0	2	2.5	3.5	4	4.5	4	3	2.5	2	1.5	Negative	Normal			
2	Normal	1/3	0	21	0	- 0	2	2.5	3.5	4.5	4	3.5	3	2	1	1	Negative	Normal			
3	Normal	8/3	0	34	+	0	4.5	5	5.5	6	6.5	5	4.5	4	2	1.5	Negative	Normal			
4	Normal	22/3	0	60	+	+	6	6.5	7	7.5	8	9	7	6	5	3.5	So-called syphilitic	Normal		Low	
5	Normal	186/3	2/3	132 130	+	+	18	18	18	18	18	18	18	18	18	18	So-called syphilitic	Normal			
6	Slender fibrin	123/3	56/3	85	+	+	4	5.5	6.5	7.5	8.5	11	13	11	9	6.5	Inconsider- ably in- creased	Markedly increased			
7	Coagulum fibrin	287/3	74/3	237	0	0	1.5	2	2.5	3.5	4	6	7	8.5	8	7.5	So-called menin- gitic	Highly in- creased		High	
8	Spherical fibrin	5/3	0	348	0	0	0.5	1.5	3	4.5	7	9.5	11	13	15	12	So-called menin- gitic	Highly in- creased			

*The color values are determined with the gold color standard: 0, red; 1 to 9, blue-red; 10, blue; 11 to 15, black.

¹The syndrome provides the location of the maximum reaction.

²The syndrome of blood effects of blood.

*The color values are determined with the gold color standard: 0, red; 1 to 9, blue-red; 10, blue; 11 to 19, blue-white; 20, colorless, complete coagulation. ^ Indicates the location of the maximum reaction.
¹The syndrome provides the "special indication," in addition to the "generally obligatory five examinations," to examine for tubercle bacilli, effects of block are absent. In this case the cerebrospinal fluid was colorless.
²The syndrome of block provides the "special indication" to examine cerebrospinal fluid collected by clasternal puncture, in which the secondary

or, in other words, of permeability of the meninges. A second criterion of importance is the presence of polymorphonuclear leucocytes in (sub-)acute inflammatory processes. A third, more universal, indicator of the state of permeability is furnished by the gold reaction. When fibrin or abundant polymorphonuclear leucocytes indicate markedly increased permeability, the gold reaction invariably yields the so-called meningitic or rather hematogenous-type of curve (see Table II; Nos. 6 to 8). The paretic or parenchymatous type (see Table II, No. 5) indicates no or little increase in permeability, while the syphilitic and negative types (Table II, No. 4, and Nos. 1 to 3) indicate normal permeability.* These three criteria make the use of other special tests for permeability unnecessary.

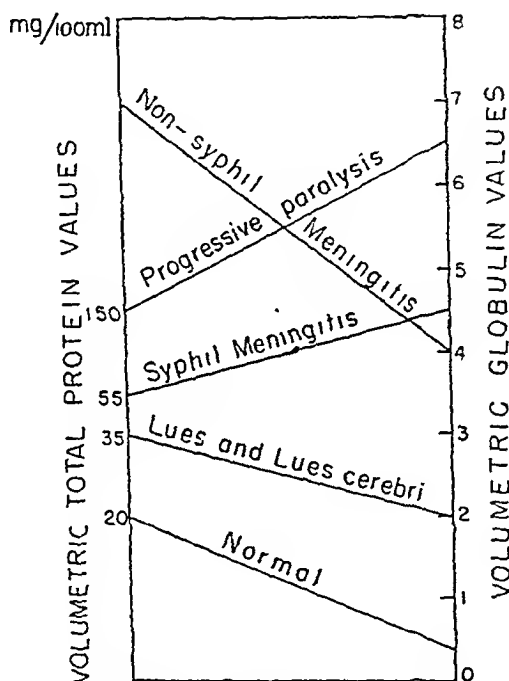


Fig. 3.—Schematic presentation of albumin-globulin ratios from Kafka.⁴

It is, of course, self-evident that the albumin-globulin ratio in cerebrospinal fluids does not depend entirely on the presence of blood proteins. Under conditions of normal permeability, the albumin-globulin ratio is affected by the intrameningeal (nonhematogenous) production of protein. The determination of the albumin-globulin ratio, however, is not necessary in routine examinations. High albumin-globulin ratios merely confirm data already provided by other means. Low ratios are encountered in normal conditions and in chronic neurosyphilis. The values obtained with chemical methods, however, are technically so unreliable and so overlapping that they are seldom characteristic for any group.

*These designations have been widely adopted; however, since the terminology may be misleading, it is recommended that the curves be designated as A, B, C, and D: that is, finding within normal limits should be classified as A; those giving evidence of chronic productive inflammatory changes, as B; hematogenous types of curve, as C; and those showing evidence of parenchymatous degeneration, as D.

More correct values of albumin-globulin ratios in cerebrospinal fluids were obtained by Kabat and associates¹⁷ with electrophoresis. Unfortunately, this method cannot be used routinely, because it requires about 70 c.c. of cerebrospinal fluid. However, it opens new possibilities in diagnosis that seem to lie not in the determination of albumin-globulin ratios but in the determination of the single protein fractions. Present indications are that these fractions in cerebrospinal fluids can be determined with specific antisera. This procedure would be economical since no fractionation would be required. Experiments are now in progress with two fractions, albumin and gamma globulin. The albumin-globulin ratio can be determined in cerebrospinal fluids by the method used for sera by Goettseh and Kendall.¹⁸ The determination of gamma globulin with values similar to those found by Kabat seems, however, to promise more significant progress in the diagnostic examination of cerebrospinal fluid.

SUMMARY AND CONCLUSIONS

Current methods of determining the albumin-globulin ratio in cerebrospinal fluid are markedly inferior to those for blood. The albumin-globulin ratio in cerebrospinal fluid varies widely because of the amount of blood proteins that may be present. Even crude qualitative methods can demonstrate some differences, provided the protein content is high. In low protein concentrations, which prevail in neurosyphilis, misleading results are obtained by using 50 per cent saturation with ammonium sulfate to precipitate the globulin. With methyl alcohol, it is possible to secure consistent results in normal cerebrospinal fluids and to determine albumin-globulin ratios in various pathologic conditions.

Evaluation of the albumin-globulin ratio should, however, be based on a systematic interpretation of the results of various laboratory tests. An attempt to correlate an isolated finding directly with clinical conditions is, as a rule, misleading. It should be interpreted as an integral part of a complete syndrome. The application of this basic principle indicates that determination of the albumin-globulin ratio in cerebrospinal fluids provides, chiefly, information regarding the permeability of the meninges, which is more conclusively indicated by other procedures.

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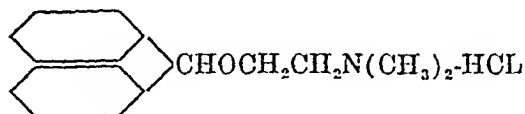
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THE INFLUENCE OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL) UPON NORMAL PERSONS AND UPON THOSE SUFFERING FROM DISTURBANCES OF THE AUTONOMIC NERVOUS SYSTEM

PRELIMINARY REPORT

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DIMETHYLAMINOETHYL benzhydryl ether hydrochloride, an alkamine benzhydryl ether, the structural formula of which is given, has been shown to possess antispasmodic and antihistamine activity of high degree.¹⁻⁴



Chemically, this drug fails to resemble other agents employed clinically because of similar properties. Moreover, the present studies suggest that it lacks some of the unpleasant and at times harmful effects of such drugs. For these reasons, it has seemed important to observe the action of the drug in a relatively wide range of dosage upon the various functions and systems of the body, both in normal subjects and in those suffering from widely diverse illnesses.

MATERIALS AND METHODS

Twenty-eight women from 25 to 65 years old, with an average age of 40.2 years, and thirty-two men from 21 to 60 years old, with an average age of 43.3 years, have served as the subjects of these tests. These subjects were divided into two major groups: (1) those who had no known disease or at least no condition in which the autonomic nervous system was directly involved, and (2) those known to have some disturbance in which the autonomic nervous system played a role. The former group consisted of sixteen women and eighteen men. Of these thirty-four subjects, eight (three women and five men) were given the complete cycle of examinations before, during the time, and after they had received dimethylaminoethyl benzhydryl ether hydrochloride, hereinafter referred to as Benadryl.* Two subjects were used to determine the effect of the drug on sleep only. Ten were used to determine the intravenous effects and blood levels only, and fourteen were chosen for the study of the local action of the drug upon the eye.

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*Generous supplies of this material under the trade name Benadryl were made available through the courtesy of Dr. E. A. Sharp, Director of Clinical Investigation, Parke, Davis & Company, Detroit, Mich.

TABLE I. EFFECTS OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE IN CERTAIN CLINICAL CONDITIONS

DIAGNOSIS	NUMBER OF PATIENTS	CHANGE IN CLINICAL CONDITION OBSERVED UNDER TREATMENT			REMARKS
		COMPLETE RELIEF	IMPROVED	UN-CHANGED	
Spastic colon	7	3	4	0	In two cases, period of time may be too short for final conclusion
Cardiospasm	2	0	0	2	Chief symptoms involved were nausea, vomiting, and pain
Other gastrointestinal neuroses	5	3	0	2	
Essential hypertension	1	0	1	0	Drop in systolic and diastolic blood pressure and disappearance of headache
Hyperthyroidism	2	0	0	2	Thyroid condition controlled prior to amine treatment; constipation and exophthalmos not relieved
Paget's disease with paralysis agitans	1	0	1	0	Tremor alone improved
Climacterium	1	0	1	0	Abdominal pains completely relieved
Dysmenorrhea, functional	2	2	0	0	Syndrome of "premenstrual tension"
Asthma	4	4	0	0	One initially seen in status asthmaticus.
Allergy to insulin, local	1	1	0	0	
Totals	26	13	7	6	

The major diagnosis encountered in each of the second group of patients is listed in Table I. In each patient of this group, there was reason to believe that a disturbance of the autonomic nervous system existed.

Details of histories, physical examinations, and laboratory tests have all been designed to afford a thorough appraisal of the status of important bodily functions and are mentioned under Results.

Unless otherwise mentioned, in "diseased" individuals 1 capsule (50 mg.) of benadryl was given three times daily.

For the eight subjects in the control group, diet was roughly regulated according to weight (Table II).

Repetition of the initial observations on these eight subjects was made at intervals of from one to two weeks before, during, and after oral administration of the drug over total periods of time ranging from six to eighteen weeks. In two of these eight control subjects, intravenous tests were performed for the purpose of observing blood levels during the immediately succeeding hours. In eight other subjects, similar observations were made following intravenous therapy.

TABLE II

WEIGHT (POUNDS)	DIET (GM.)			CALORIES
	CHO	PROTEIN	FAT	
140 or less	135	70	80	1,540
140 or 175	175	75	100	1,900
175 or more	200	95	125	2,200

Patients in whom the findings are recorded received the drug for periods of from two to eighteen weeks.

RESULTS

For purposes of clarity and comparison, data from control subjects and patients are summarized together, except in those instances where the latter differed materially from the former. In view of the nature of the study, the data are arranged under the systems of the body in so far as possible.

General Metabolism.—No alterations in weight or basal metabolic rate of either patients or control subjects could be ascribed to the direct effects of administered benadryl.

The Central Nervous System.—Both normal and "sick" subjects became more placid while receiving treatment with benadryl.

Seven of ten "normal" subjects observed that they slept better than formerly. Two of these seven subjects observed sleepiness during the day as a result of the ingestion of a single 50 mg. capsule. Among the twenty-six patients who received the drug (Table I), sleep was improved in seventeen and was unchanged in five; the status of sleep was not recorded in four subjects. A tendency toward daytime somnolence was observed by three patients. One of these had to reduce the dose of drug from 150 to 100 mg. but was later able to resume the previous dose without further unpleasant symptoms.

The Eye.—No variations in the size of the pupils under fixed conditions of lighting were observed in nine patients before and after therapy with orally administered benadryl, or during its use in daily doses ranging from 150 to 400 mg., even after the largest doses were employed for five weeks at a time.

Locally, the effects were quite different. An aqueous solution (4 per cent) was well tolerated by the conjunctiva of rabbits, but a stronger solution (5 per cent) caused hyperemia and edema. For human experiments, we used 0.5 and 1.0 per cent solutions of the drug without discomfort to the patients unless the solution had stood in a clear bottle for several weeks, after which it became irritative.

Most of the work was done with the lesser concentration. This caused an average pupillary dilatation of 1.0 mm. within fifteen minutes in nine of fourteen and of 1.8 mm. in one hour in all of the fourteen patients. An interference with accommodation always accompanied the mydriatic effect. Epinephrine hydrochloride (1 per cent solution) and benadryl (0.5 per cent solution) were applied simultaneously in six patients. Within fifteen minutes all but one patient showed a dilatation averaging slightly less than 1.0 mm. and in one hour an average dilatation amounting to 1.7 mm.; this was practically identical with that produced by the solution of the alkamine ether alone.

In fifteen trials on fourteen patients this amount of epinephrine, without the benzhydryl ether, showed no appreciable reaction in the size of the pupil even after one hour. We may conclude, therefore, that epinephrine hastened the action of the drug but did not alter its total effect.

Homatropine sulfate (1 per cent solution) and eserine sulfate (1 per cent solution) produced their characteristic mydriatic and miotic effects, respectively.

These were not influenced by the use of benadryl administration orally in doses up to 400 mg. daily in any one of the five patients tested.

The Respiratory System.—Alterations in the rate and character of the respirations were not observed in any but asthmatic individuals. In these subjects, administration of benadryl caused a decrease in respiratory rate and a rise in vital capacity to normal levels (Table III). Vital capacity was determined in all the control subjects and in two of the four patients with asthma, using the Benedict-Roth respirometer and the Dreyer formula.⁵

TABLE III. RESPIRATORY RATE AND VITAL CAPACITY IN SUBJECTS TAKING DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

SUBJECTS	DOSAGE (MG. PER DAY)	DURATION OF TREATMENT (WEEKS)	VITAL CAPACITY (PER CENT)	RESPIRATIONS (PER MINUTE)
Eight controls	0	--	90	16
	150	4	101	18
	300	3	89	17
	400	4	97	18
Two with asthma	0	--	40	28
	150	1	43	26
	300	3	85	18
	400	5	84	17

The Cardiovascular System.—Following the oral administration of the alkamine ether, *dizziness and blurring of vision* were the only symptoms suggesting cardiovascular disturbances which could have been attributed with reasonable certainty to the drug. Even though 100 mg. were given four times daily, these symptoms were not seen in any subjects of the control group; however, they were observed in two patients with a gastrointestinal neurosis and in one patient with dysmenorrhea. Irregularities in the circulation, rather than a direct toxic action upon nervous tissue, were thought to be responsible, since two of the three patients showed an associated orthostatic hypotension (see paragraph on blood pressure). On the other hand, orthostatic hypotension did not necessarily produce dizziness or blurring of vision, since six subjects developed this condition without any associated symptoms.

The size of the heart and of the liver showed no clinically detectable alteration even after prolonged treatment (up to eighteen weeks) with as much as 100 mg. of dimethylaminoethyl benzhydryl ether hydrochloride four times daily by mouth. Teleroentgenograms of the heart and lungs of all subjects revealed no changes due to the action of the drug.

In thirty-six subjects given 300 mg. or less of the drug daily, no alterations in the pulse rate were observed, except in the group of patients with asthma.

Lowering of the systolic blood pressure was observed in three of forty-six subjects in whom serial examinations were made. In two of these patients who were taking 150 mg. daily, dizziness of mild degree was noted. The third subject was given 200 mg. daily without untoward subjective symptoms; the average degree of depression was 15 mm. of mercury. Orthostatic hypotension was observed in a total of six subjects. Three of the six were control subjects who developed the condition while taking 300 mg. or more daily. One patient with

cardiospasm and one with dysmenorrhea, each receiving 300 mg. daily, and one individual suffering from hyperthyroidism and dyschesic constipation, who was given 200 mg. daily, completed the group in which orthostatic hypotension was observed. In all but one individual, these changes in the blood pressure reverted to normal within several days after discontinuing the drug. This individual was a control subject who had received 400 mg. of the alkamine ether daily for approximately three and one-half weeks and had previously taken smaller doses over a total period of five weeks. Two months after cessation of therapy, an orthostatic hypotension persisted which may not have been due to the drug, since pressures were not checked in all positions prior to beginning its administration.

After the oral administration of as much as 400 mg. of benadryl daily, no appreciable changes could be demonstrated in the *capillary loops* of the fingernail beds of the five "normal" subjects and of the one asthmatic patient studied.

The *Rumpel-Leede phenomenon* was routinely negative in subjects using the drug.

The *circulation times and capillary permeability* were determined in six subjects by the fluorescein technique of Lange and Boyd.⁶ Circulation times were apparently not influenced by the drug in doses up to 400 mg. daily by mouth (Table IV). Capillary permeability was decreased by large doses of the drug (Table IV).

TABLE IV. DETERMINATION OF CAPILLARY PERMEABILITY AND CIRCULATION TIME BY FLUORESCIN TECHNIQUE IN EIGHT CONTROL SUBJECTS BEFORE, DURING, AND AFTER ADMINISTRATION OF DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

NUMBER OF SUBJECTS	DOSAGE (MG. PER DAY)	DURATION TREATMENT (WEEKS)	C. T.* (SEC.)		CAPILLARY PERMEABILITY (PER CENT NORMAL)†	
			RANGE	AV.	RANGE	AV.
8	0	--	27 to 42	33	+ 2 to -21	-11
8	150	4.3	28 to 36	30	+ 6 to -39	-20
2	200	3.0	26 to 46	36	- 4 to -20	- 9
6	300	3.0	28 to 36	33	-24 to -41	-33
4	400	3.5	28 to 44	38	-24 to -61	-38

*Arm to leg.

†Calculated from the average mean curve. Variations of plus or minus 25 per cent are still within the normal range.

Electrocardiographic tracings showed no changes as a result of the use of the alkamine ether.

When a solution of *dimethylaminoethyl benzhydryl ether hydrochloride* was given *intravenously*, three of ten subjects developed "reactions." One normal subject had taken the drug orally in divided daily doses of 150 mg. for one week, 300 mg. daily for two weeks, and 400 mg. daily for four weeks, without ill effects. Four hours after an additional dose of 20 mg. given *intravenously*, this subject complained of weakness, headache, "seasickness," and drowsiness, all associated with extreme pallor, but no sweating. Despite the continuance of the drug orally, all these symptoms had disappeared by the following day. A second subject of the "normal" group had completed three months of treatment with oral doses up to 400 mg. of the amine daily. Eleven days later 30

mg. were given intravenously. Fifteen minutes thereafter, the subject complained of dizziness with a sensation of falling toward the left. The entire reaction disappeared in one hour without treatment. The third reaction to the intravenous injection of the alkamine ether occurred in a patient with controlled thyrotoxicosis who had not previously received the drug. Almost immediately after a dose of 30 mg., the patient developed a severe chill associated with sweating, headache, low back pain, and a slight decrease in temperature (to 97.6° F.) but with no change in either pulse rate or blood pressure. The remaining seven subjects, to none of whom had the drug been administered previously, showed no untoward reaction of any kind to the intravenous injection of as much as 30 mg. Such doses appreciably altered neither the respiratory rate nor the cardiac function, nor did they produce any changes either in degree of perspiration or in the color of the skin.

The Gastrointestinal System.—No gross change was observed in the quality or quantity of *salivary secretion* in any of the eight control subjects when as much as 400 mg. of the benzhydryl ether were taken daily. In one patient with gastrointestinal neurosis, in whom 150 mg. of the drug were given daily, increased thirst and dryness of the mouth were subjectively noted, but a second patient with similar diagnosis and treatment experienced mild salivation.

Mouth-anus time as determined in six control subjects through the ingestion of charcoal (1 Gm.) was not altered by the drug. *Bowel habits* were unchanged by the drug in all control subjects and in all the patients except the seven who suffered from a spastic colon. Follow-up barium enemas revealed a decrease in the spastic condition in the four patients with spastic colon in whom such an examination was possible.

Seventeen of the twenty-six patients complained of abdominal pain when first seen (Table V). Under treatment with the drug, ten of the seventeen showed partial or complete relief of such distress. In two of the remaining seven patients, sufficient time has not elapsed to draw final conclusions, although usually the drug acts quickly. The pain of three patients was not relieved. Details are shown in Table V.

In regard to *gastric acidity*, there was a marked decrease in the amount of both free and combined acid as a result of the administration of the benzhydryl ether in four of five control subjects who showed free acid prior to the use of benadryl. Moreover, there was little or no response to histamine. The details

TABLE V. VARIATIONS IN ABDOMINAL PAIN IN PATIENTS TREATED WITH DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDROCHLORIDE

DIAGNOSIS	NUMBER OF PATIENTS	COMPLETE RELIEF	PAIN		FOLLOW-UP INSUFFICIENT
			IMPROVED	NO CHANGE	
Spastic colon	7	4	1	2	0
Cardiospasm	2	0	0	2	0
Other gastrointestinal neuroses	5	1	1	1	2
Climacterium with reflex gastrointestinal disturbances	1	0	1	0	0
Dysmenorrhea, functional.	2	2	0	0	0

are included in Figs. 1 to 4. The fifth patient showed a definite drop in the acid values, but the dosage and duration of treatment were not sufficient to develop the full effects of the drug.

Three of eight patients with gastrointestinal disturbances, upon whom fractional gastric analyses were performed, showed an anacidity except after histamine stimulation, presumptively as a result of old age. The acid values following histamine injection were markedly decreased in all of these by the

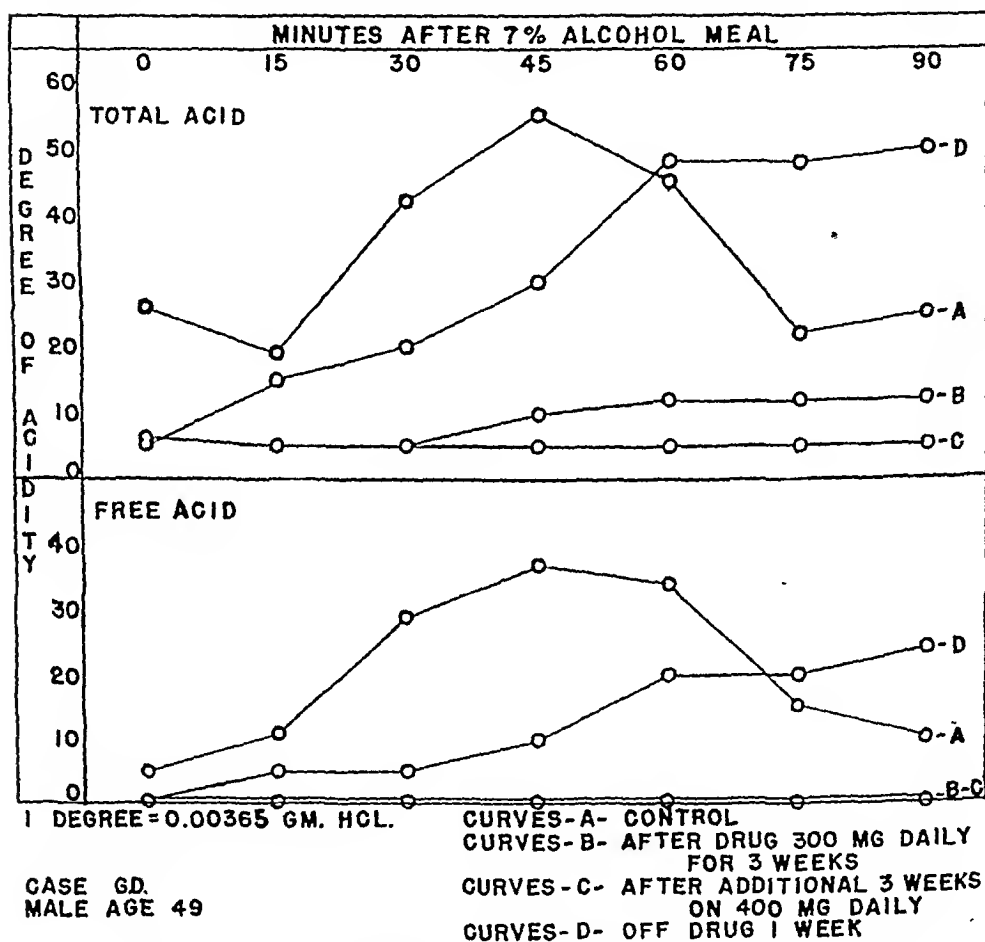
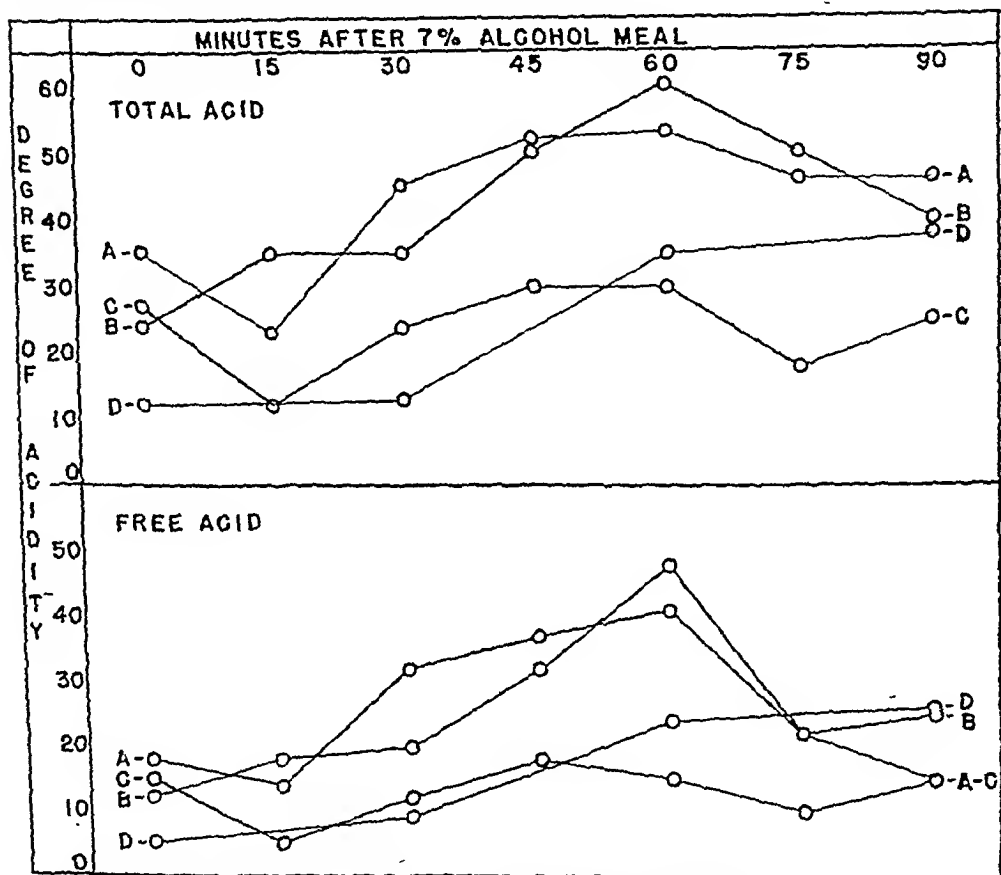


Fig. 1.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

use of the benzhydryl ether in doses of from 150 to 200 mg. daily for periods of two, three, and four weeks, respectively. The other five patients exhibited responses in the gastric secretion similar to those observed in the control subjects.

Renal Function.—No variation in urinary volume was observed as a result of the use of dimethylaminoethyl benzhydryl ether hydrochloride, either in the control subjects or in patients. In six subjects, Fishberg's modification of Volhard's concentration and dilution tests⁷ failed to show any variations from the

normal. Repeated routine urinalyses were likewise uninformative. Urea nitrogen and nonprotein nitrogen determinations, performed upon the blood of all subjects before, during, and after treatment, remained consistently within normal limits. No indication for further investigation of renal function arose during the course of the experiments.



1 DEGREE = 0.00365 GM. HCL.

CURVES-A- CONTROL
 CURVES-B- AFTER DRUG 300MG DAILY
 FOR 3 WEEKS
 CURVES-C- AFTER ADDITIONAL 2.5
 WEEKS ON 400MG DAILY
 CURVES-D- OFF DRUG 2 WEEKS

CASE Q.R.
 MALE AGE 50

FIG. 2.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

Skin.—Following the oral, intravenous, and topical application of the benzhydryl ether, there was no demonstrable alteration in skin color or temperature, but definite influence upon capillary permeability (see page 564) was seen and the histamine-wheal reaction was strikingly altered. Dimethylaminoethyl benzhydryl ether hydrochloride was applied to the volar surface of the forearm as a 1 per cent ointment in a cholesterol base, using first an aqueous

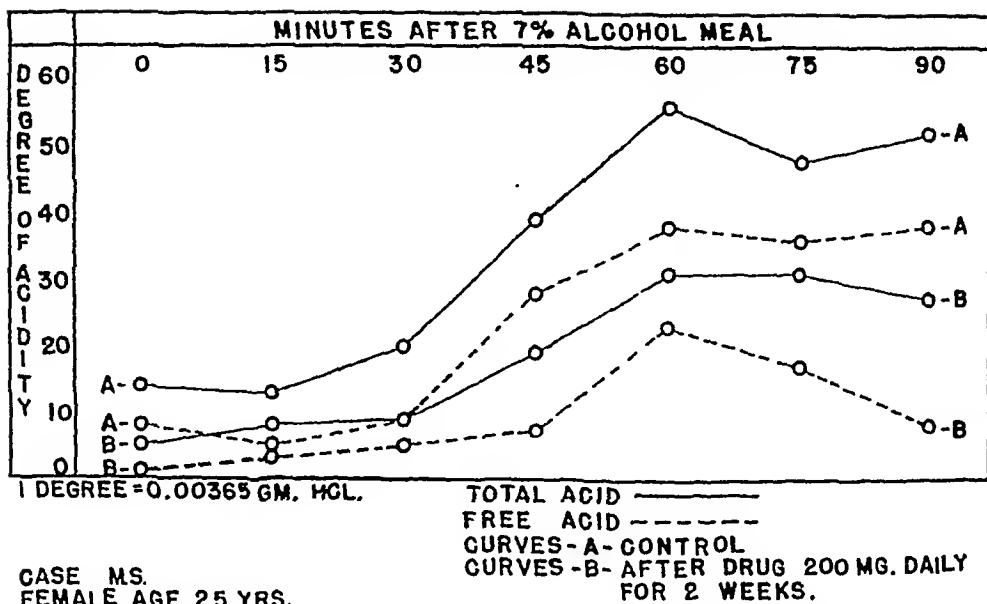


Fig. 3.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

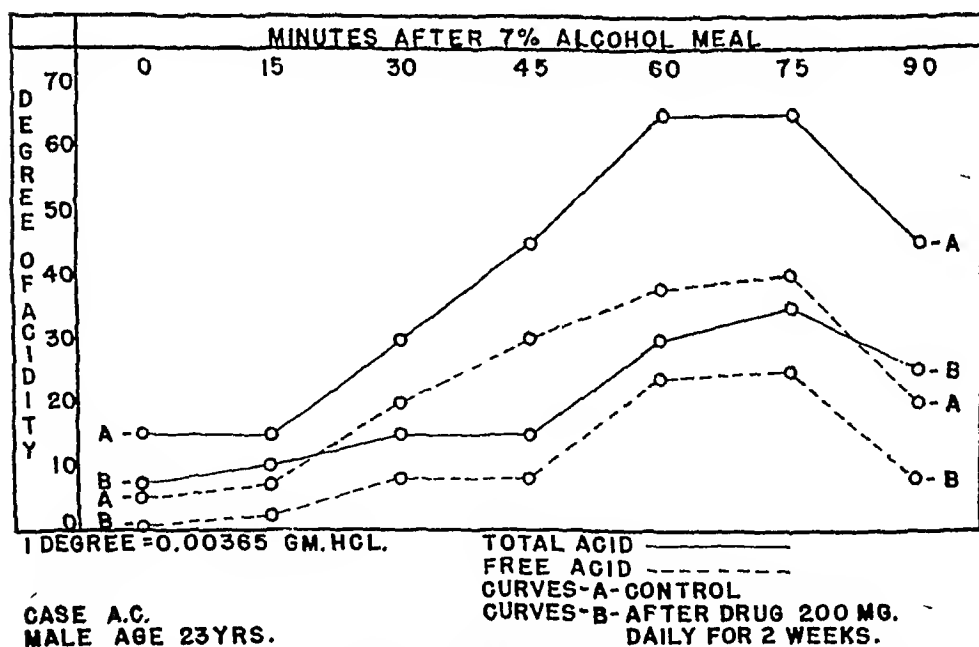


Fig. 4.—Fractional gastric analysis in relation to treatment with dimethylaminoethyl benzhydryl ether hydrochloride.

and later a nonaqueous phase. One hour later histamine was applied in the usual way to both forearms. In no instance was there a difference in the size or intensity of the histamine wheal and erythema as a result of the previously applied ointment. However, a similarly prepared ointment of 5 per cent strength completely suppressed the response to histamine.

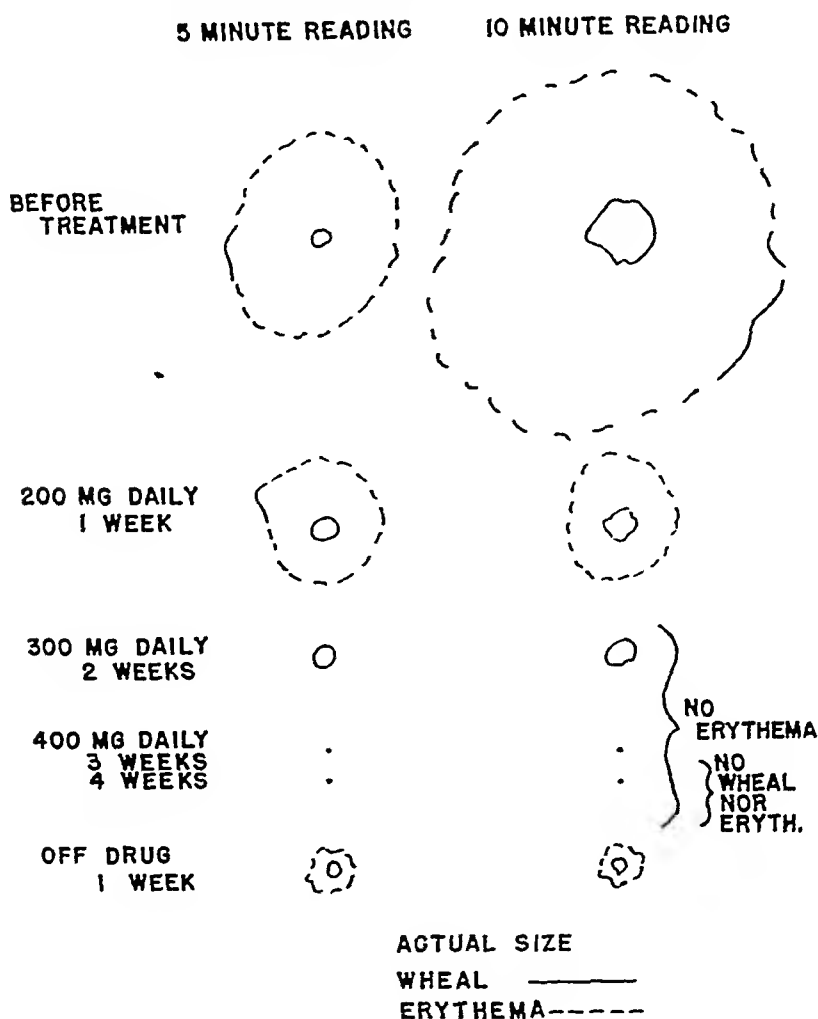


Fig. 5.—Dermal reaction to histamine, five and ten minutes after application, respectively, in patient (O. K.) before, during, and after treatment with dimethylaminoethyl benzhydryl ether hydrochloride (two-thirds actual size).

The average results of skin sensitivity to histamine applied intradermally in five control subjects according to a method previously described⁸ are noted in Figs. 5 to 7. The degree of reaction in each instance is recorded as a percentage of the initial response, or response of the "fore" period. As was to be expected, sensitivity to histamine varied considerably from patient to patient. For instance, in two subjects the sensitivity was not sufficiently great prior to treatment with the drug to justify continuation of study in them. Because of

these facts, each of the five subjects mentioned was made his own control. It is clear from Fig. 5 that the average therapeutic dose of 50 mg. of the benzhydriyl ether administered three times daily, was capable of producing an appreciable reduction in the severity of the histamine reaction. A further striking decrease occurred when 300 mg. per day were given, beyond which additional increments in dosage had little, if any greater, effect (up to 400 mg. daily tried).

These skin reactions closely paralleled the behavior of the systemic reactions to the histamine injections which were used in carrying out the fractional

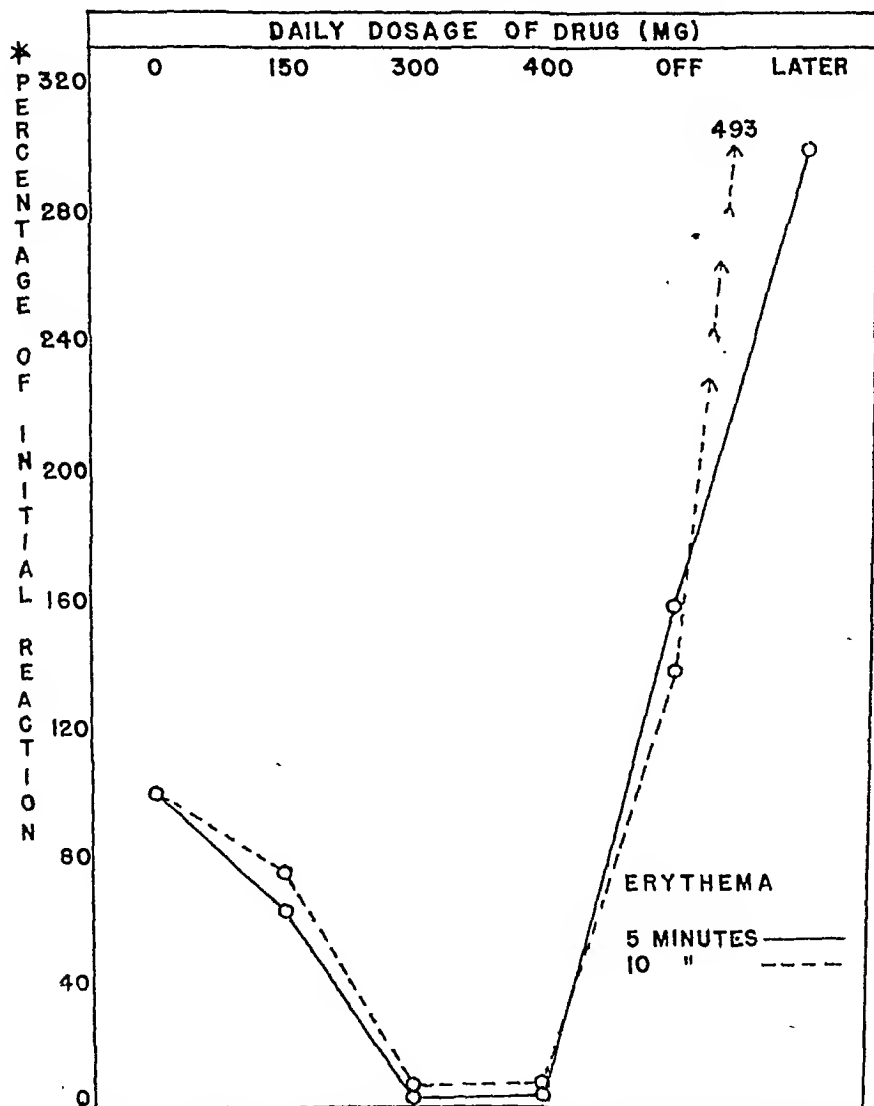


Fig. 6.—Influence of varying dosage of dimethylaminocetyl benzhydriyl ether hydrochloride upon skin sensitivity to histamine (average results in five subjects).

*The degree of reaction in the "fore" period has been placed at 100 for each subject and subsequent values were derived as a percentage of this value.

gastric analyses (0.25 mg.). Two "normal" subjects obtained such a reaction in the "fore" period, which was completely abolished by the use of 150 mg. of the drug daily for as little as ten days. Reactions were ameliorated in one asthmatic patient by the use of 300 mg. of the drug and completely abolished in the same patient by 400 mg. daily.

Blood.—The erythrocyte and leucocyte counts, hemoglobin determinations, and differential white counts performed in all subjects before and in most of them during treatment with dimethylaminoethyl benzhydryl ether hydrochloride failed to show any significant changes. Hematocrit readings and the speed of sedimentation of the erythrocytes noted in eight control subjects and in four patients were comparable in each individual instance before, during, and after treatment.

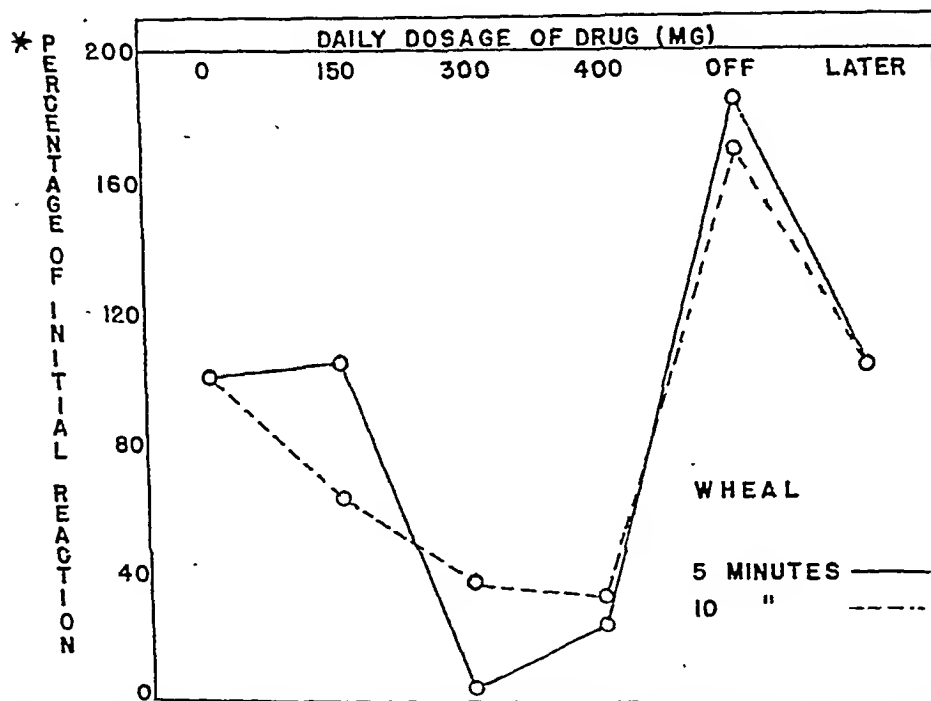


Fig. 7.—Influence of varying dosage of dimethylaminoethyl benzhydryl ether hydrochloride upon skin sensitivity to histamine (average results in five subjects).

*See Fig. 6 for information.

In all sixty subjects, the blood nonprotein nitrogen, urea nitrogen, creatinine, and glucose were within normal range before, during and after therapy with the alkamine ether. Total proteins, the albumin-globulin ratio, total and free cholesterol, alkaline phosphatase, the icteric index, and the van den Bergh reaction, and the cephalin flocculation were determined in sixteen subjects by a method previously described.⁹ No significant variations occurred in relation to treatment with benadryl.

The Therapeutic Activity of Dimethylaminoethyl Benzhydryl Ether Hydrochloride.—The responses of twenty-six patients who have received dimethyl-

aminoethyl benzhydryl ether hydrochloride are tabulated in Table I. These suggest the probable future trends of therapy with benadryl.

Untoward Reactions.—An undue sense of *fatigue and sleepiness* occurred in approximately 15 per cent of thirty-six subjects. *Dizziness and blurring of vision* was noted in three subjects. A transient partial amnesia occurred in one subject who received 400 mg. of the drug in a single dose.

DISCUSSION

Untoward Reactions.—In the acceptance or rejection of any agent for therapeutic purposes, the nature, severity, and duration of untoward reactions must be prime considerations. The unpleasant symptoms produced by dimethylaminoethyl benzhydryl ether hydrochloride have been of mild degree, sometimes disappearing while the medication was continued. They have always been relieved promptly by discontinuing the drug or by decreasing the amounts administered. Indeed, when the amine is given orally, their occurrence is extremely rare. Other workers have had a similar experience.

Nature of the Action of Benadryl.—The vigorous antihistamine action of a series of benzhydryl alkamine ethers, to which dimethylaminoethyl benzhydryl ether hydrochloride belongs, has been amply demonstrated in animals^{1, 2, 4} and in human beings.³ The present studies further illustrate the clinical applicability of these effects, since patients with asthma, functional dysmenorrhea, and urticaria have been successfully treated.

The role of histamine in the production of functional dysmenorrhea may be questioned, but the relation of certain types of this condition to the activity of the autonomic nervous system seems to be rather clear.¹⁰⁻¹³ The influence of dimethylaminoethyl benzhydryl ether hydrochloride upon the clinical syndrome of spastic colon is not so clear, although here again a direct antihistamine effect may be operative,¹⁴ particularly in view of the fact that histamine may be readily synthesized by the intestine.¹⁵ Further evidence for the antihistamine nature of the action of the drug is its ability to lower gastric acidity. Current concepts accord histamine a normal role in the production of hydrochloric acid by the stomach.^{14, 16, 17} It seems logical to assume from the present data that the decrease or even disappearance of free hydrochloric acid from the gastric juice of patients receiving the alkamine ether is due to its retarding effect upon that activity of histamine.

The tendency for histamine to cause tissue exudation may be related to an alteration in the permeability of the capillaries. If that is so, then the decrease in permeability produced by the benzhydryl ether in the present series of experiments would be additional proof of an antagonism to histamine.

When the antispasmodic action of dimethylaminoethyl benzhydryl ether hydrochloride is compared with that of papaverine, its potency is "approximately 650, 50, and 1.3 times that of the latter drug relative to antagonism of histamine, acetylcholine, and barium chloride, respectively."¹⁴ Therefore, while the antihistamine action is by far the most striking feature of the action of dimethylaminoethyl benzhydryl ether hydrochloride, its direct effect upon the autonomic nervous system deserves consideration.

Topical application of the drug to the eye results in pupillary dilatation. This appears to be an atropine-like or at least a parasympathetic depressant, effect. The response is hastened but not increased by the simultaneous use of epinephrine.

The failure of the drug to accelerate the pulse, except in the largest doses, or to increase blood pressure is further evidence that the direct sympathomimetic influence is slight or nonexistent, while the antihistamine effect is great. This makes the drug particularly desirable as an antispasmodic in asthma, where too often cardiac acceleration and even failure exist before treatment is begun. The mild quieting action of the drug is of value in connection with all of the gastrointestinal neuroses, the urticarias and, indeed, any allergic condition; such sedative property is not shared by epinephrine or any of its chemically related substitutes.

We may conclude that dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) is apparently an effective agent for use in allergic disease. Obvious advantages over other agents ordinarily employed for such conditions are the low toxicity and the freedom from unpleasant cardiovascular and nervous symptoms. Complications due to treatment include somnolence and orthostatic hypotension, either of which may necessitate a decrease in dosage, but these conditions almost never completely preclude the successful use of the drug.

SUMMARY

1. The effects of dimethylaminoethyl benzhydryl ether hydrochloride have been studied in normal persons, and in those with widely diverse diseases, most of which have been associated with some disturbance of the autonomic nervous system.

2. Results of the following determinations were not abnormally altered in any of the subjects by the administration of the drug: basal metabolism, circulation time, renal function, erythrocyte and leucocyte counts, hemoglobin, differential white counts, hematocrit readings, blood urea nitrogen, creatinine, glucose, proteins, cholesterol, alkaline phosphatase, icteric index, van den Bergh reaction, and cephalin flocculation.

3. An increase in weight occurred in four patients as a result of improvement in their general condition.

4. The majority of subjects showed no change in pulse rate or in blood pressure. Orthostatic hypotension was observed in six subjects. A slight elevation in pulse rate was seen in five patients taking 400 mg. of dimethylaminoethyl benzhydryl ether hydrochloride daily for relatively long periods of time.

5. Pupillary dilatation, accelerated in its appearance but not increased in its amplitude by epinephrine, was observed when the drug was instilled into the conjunctivae.

6. Vital capacity and respiratory rate were uninfluenced by the drug, unless initially lowered as a result of asthma.

7. Capillary permeability was moderately decreased by prolonged treatment with large doses of the alkamine ether compound.

8. Three of ten subjects receiving the drug intravenously developed mild transient reactions.

9. Salivary secretion was apparently not influenced by the range of dosage employed throughout these studies. Both free and total gastric acidity were strikingly decreased. The mouth-anus time was not altered in the normal subjects. Abdominal pain was relieved in ten of seventeen patients.

10. The degree of reduction in the size of the histamine skin reactions was roughly proportional to the daily dose of drug administered. For a brief period after the drug was discontinued, the response to histamine was increased.

11. Definite therapeutic effect was obtained from the drug in certain gastrointestinal neuroses, asthma, functional dysmenorrhea, and dermal allergy.

12. Untoward reactions to benadryl were of mild degree and of infrequent occurrence. They included dizziness, blurring of vision, weakness, and somnolence. Each disappeared promptly upon discontinuing the drug or decreasing the dose.

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APPLICATION OF THE Rh BLOOD TYPES AND Hr FACTOR IN DISPUTED PARENTAGE

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NOW that the theory of multiple allelic genes¹ for the heredity of the Rh blood types is firmly established,^{2, 3} these tests are being applied as a routine alongside of the ordinary blood grouping, subgrouping, and M-N tests in cases of disputed parentage. Sufficient evidence has also accumulated to justify the routine use of the standard Hr factor (Hr'),^{4, 5} although sera corresponding to the factor Hr'' recently described in England⁶ are not yet generally available. When discussing the application of the Rh blood types and standard Hr factor in the past, our practice has been merely to give the rules of inheritance, from which the possibilities for any particular mating could be derived. The proper application of these laws requires considerable genetic and serologic knowledge, however, and lawyers, in particular, have requested detailed tables listing all the possible matings for reference use. The tables are far more elaborate than in the case of the blood groups and M-N types, and some of the matings involve rare types which will hardly ever be encountered in practical work. Nevertheless, in view of the need for such a complete set of tables, this paper was prepared.

THE EIGHT Rh BLOOD TYPES

As shown in Table I, with the aid of the three varieties of Rh antisera, anti-Rh₀, anti-Rh' and anti-Rh'', eight types of blood can be distinguished. The factor Rh₀ was so designated because it has a special position in relation to the other two Rh factors. Thus, if the reactions of the anti-Rh₀ serum are disregarded, a scheme of four types results which is analogous serologically and genetically (except for the rare Rh₂ factor) to the four Landsteiner blood groups.

When selecting the designations of the eight Rh blood types, I was guided by the principle of maximum simplicity without ambiguity.⁷⁻¹⁰ Thus, blood not agglutinable by any of the three Rh antisera is said to be Rh negative, or, more simply, is said to belong to type rh. Types Rh', Rh'', Rh'Rh'', and rh₀ are named after the antisera with which these bloods react. Type Rh₁ reacts with the two antisera, anti-Rh' and anti-Rh₀, and Rh₁ is merely an abbreviation for Rh₀'; similarly, Rh₂ is short for Rh₀''. Types Rh₁ and Rh₂ are so named, instead of Rh₀Rh' and Rh₀Rh'', respectively, to indicate that the two factors Rh₀ and Rh' in the former, and the factors Rh₀ and Rh'' in the latter behave like "partial" antigens in complex agglutinogens which are, as a rule, hereditarily transmitted as units by the corresponding genes, R'

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TABLE I. CLASSIFICATION OF EIGHT Rh BLOOD TYPES

BLOOD CONTAINING FACTOR Rh _o				BLOOD NOT CONTAINING FACTOR Rh _o			
DESIGNATION OF TYPES*	REACTIONS WITH SERA			DESIGNATION OF TYPES*	REACTIONS WITH SERA		
	ANTI-Rh'	ANTI-Rh''	ANTI-Rh _o		ANTI-Rh'	ANTI-Rh''	ANTI-Rh _o
rh _o	-	-	+	rh	-	-	-
Rh ₁	+	-	+	Rh'	+	-	-
Rh ₂	-	+	+	Rh''	-	+	-
Rh ₁ Rh ₂	+	+	+	Rh/Rh''	+	+	-

*Type rh was formerly designated Rh negative; type Rh₁ contains the two factors Rh_o and Rh', the name Rh₁ being short for Rh_o'; similarly, Rh₂ is short for Rh_o'', and Rh₁Rh₂ is short for Rh_o'Rh_o''.

and R². Type rh_o is printed with a small "r" to indicate that this type bears a similar relation to types Rh₁, Rh₂, and Rh₁Rh₂ to that which type rh bears to Rh', Rh'', and Rh/Rh''. In giving the names of the Rh types verbally, however, it is not necessary to say small "r" or large "r," as the ease may be, because the qualifying subscripts and superscripts will prevent any ambiguity.

According to the theory of six allelic genes,¹ the eight Rh blood types are hereditarily transmitted by means of a series of allelic genes, *r*, *R'*, *R''*, *r^o*, *Rⁱ*, and *R²*. It will be noticed that in order to avoid confusion between the names of the Rh genes and genotypes on the one hand, and the Rh agglutinogens and phenotypes on the other, the letter "h" is omitted, and italics are used in the designations of the former. The uniform use of qualifying superscripts in the gene symbols is necessary in order to conform with the practice of geneticists when naming genes belonging to a single allelic series. Under the six-gene theory, twenty-one genotypes are possible, and the way in which these correspond with the eight standard Rh types is shown in Table II.

TABLE II. EIGHT Rh TYPES AND THEIR TWENTY-ONE GENOTYPES*

Rh BLOOD TYPES	POSSIBLE GENOTYPES
rh	<i>rr</i>
Rh'	<i>R'R'</i> and <i>R'r</i>
Rh''	<i>R''R''</i> and <i>R''r</i>
Rh/Rh''	<i>R'R''</i>
rh _o	<i>rr^o</i> and <i>r^or</i>
Rh ₁	<i>RⁱRⁱ</i> , <i>RⁱR'</i> , <i>Rⁱr</i> , <i>Rⁱr^o</i> , and <i>Rⁱr^o</i>
Rh ₂	<i>R²R²</i> , <i>R²R''</i> , <i>R²r</i> , <i>R²r^o</i> , and <i>R²r^o</i>
Rh ₁ Rh ₂	<i>RⁱR²</i> , <i>RⁱR''</i> , and <i>RⁱR²</i>

*Does not take into account the rare *R³* gene or the rare intermediate genes.

When the Rh blood types are applied in cases of disputed parentage, the practical consequences of the theory of six allelic genes can be summarized in the following two rules: (1) The factors Rh_o, Rh', and Rh'' cannot appear in the blood of a child unless present in the blood of one or both parents. (2) When either parent belongs to type Rh₁Rh₂ or Rh/Rh'', no child of type rh or rh_o can occur. Similarly, parents of type rh or rh_o cannot have children of type Rh₁Rh₂ or Rh/Rh''.

In studies of the eight Rh blood types on a total of 300 families with 620 children, we have not encountered a single exception to these two rules of heredity.^{2, 3, 10} The theory of six allelic genes is also supported by statistical studies on the distribution of the Rh blood types in the general population.¹¹

TABLE III. EIGHT Rh BLOOD TYPES IN DISPUTED PARENTAGE

MATING	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE
rh × rh	rh	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , rh ₀ , Rh', Rh'', and Rh'Rh''	
rh × Rh'	rh and Rh'	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , rh ₀ , Rh'', and Rh'Rh''	
rh × Rh''	rh and Rh''	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , rh ₀ , Rh', and Rh'Rh''	
rh × Rh'Rh''	Rh' and Rh''	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , and rh ₀	Rh'Rh'' and rh
rh × rh ₀	rh and rh ₀	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , Rh', Rh'', and Rh'Rh''	
rh × Rh ₁	Rh ₁ , Rh', rh ₀ , and rh	Rh ₂ , Rh'', Rh ₁ Rh ₂ , and Rh'Rh''	
rh × Rh ₂	Rh ₂ , Rh'', rh ₀ , and rh	Rh ₁ , Rh', Rh ₁ Rh ₂ , and Rh'Rh''	
rh × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', and Rh''		Rh ₁ Rh ₂ , Rh'Rh'', rh ₀ , and rh
Rh' × Rh'	rh and Rh'	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , rh ₀ , Rh'', and Rh'Rh''	
Rh' × Rh''	Rh', Rh'', Rh'Rh'', and rh	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , and rh ₀	
Rh' × Rh'Rh''	Rh', Rh'', and Rh'Rh''	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , and rh ₀	rh
Rh' × rh ₀	Rh ₁ , Rh', rh ₀ , and rh	Rh ₂ , Rh'', Rh ₁ Rh ₂ , and Rh'Rh''	
Rh' × Rh ₁	Rh ₁ , Rh', rh ₀ , and rh	Rh ₂ , Rh'', Rh ₁ Rh ₂ , and Rh'Rh''	
Rh' × Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh'', Rh ₁ Rh ₂ , Rh'Rh'', rh ₀ , and rh		
Rh' × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh'', Rh ₁ Rh ₂ , and Rh'Rh''		rh ₀ and rh
Rh'' × Rh''	Rh'' and rh	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , rh ₀ , Rh', and Rh'Rh''	
Rh'' × Rh'Rh''	Rh', Rh'', and Rh'Rh''	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , and rh ₀	rh
Rh'' × rh ₀	Rh ₂ , Rh'', rh ₀ , and rh	Rh ₁ , Rh', Rh ₁ Rh ₂ , and Rh'Rh''	
Rh'' × Rh ₁	Rh ₁ , Rh ₂ , Rh', Rh'', Rh ₁ Rh ₂ , Rh'Rh'', rh ₀ , and rh		
Rh'' × Rh ₂	Rh ₂ , Rh'', rh ₀ , and rh	Rh ₁ , Rh', Rh ₁ Rh ₂ , and Rh'Rh''	
Rh'' × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh'', Rh ₁ Rh ₂ , and Rh'Rh''		rh ₀ and rh

(Continued on next page.)

TABLE III—CONT'D

MATING	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE
Rh'Rh" × Rh'Rh"	Rh', Rh", Rh'Rh"	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , and rh ₀	rh
Rh'Rh" × rh ₀	Rh ₁ , Rh', Rh ₂ , and Rh"		Rh ₁ Rh ₂ , Rh'Rh", rh ₀ and rh
Rh'Rh" × Rh ₁	Rh ₁ , Rh', Rh ₂ , Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh
Rh'Rh" × Rh ₂	Rh ₁ , Rh', Rh ₂ , Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh
Rh'Rh" × Rh ₁ Rh ₂	Rh ₁ , Rh', Rh ₂ , Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh
rh ₀ × rh ₀	rh ₀ and rh	Rh ₁ , Rh ₂ , Rh ₁ Rh ₂ , Rh', Rh", and Rh'Rh"	
rh ₀ × Rh ₁	Rh ₁ , Rh', rh ₀ , and rh	Rh ₂ , Rh", Rh ₁ Rh ₂ , and Rh'Rh"	
rh ₀ × Rh ₂	Rh ₁ , Rh", rh ₀ , and rh	Rh ₁ , Rh', Rh ₁ Rh ₂ , and Rh'Rh"	
rh ₀ × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', and Rh"		Rh ₁ Rh ₂ , Rh'Rh", rh ₀ , and rh
Rh ₁ × Rh ₁	Rh ₁ , Rh', rh ₀ , and rh	Rh ₂ , Rh", Rh ₁ Rh ₂ , and Rh'Rh"	
Rh ₁ × Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh", Rh ₁ Rh ₂ , Rh'Rh", rh ₀ , and rh		
Rh ₁ × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh
Rh ₂ × Rh ₂	Rh ₁ , Rh", rh ₀ , and rh	Rh ₁ , Rh', Rh ₁ Rh ₂ , and Rh'Rh"	
Rh ₂ × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh
Rh ₁ Rh ₂ × Rh ₁ Rh ₂	Rh ₁ , Rh ₂ , Rh', Rh", Rh ₁ Rh ₂ , and Rh'Rh"		rh ₀ and rh

However, that rare but valid exceptions to the second rule of heredity may eventually be encountered follows from the discovery of the rare R^z gene by Murray, Race, and Taylor¹² and of the so-called intermediate genes by me.¹³ Accordingly, when applying the Rh tests in disputed parentage, while any contradiction to the first rule of heredity should be considered reliable proof of nonparentage, a contradiction to the second rule may be only considered as evidence that parentage is very improbable.

In Table III, are listed all the possible matings of the eight Rh blood types, giving the types possible among the children under the six-gene theory. In the third column of Table III are given the types which cannot occur in the children based on the first rule; in such instances parentage is definitely excluded. In the last column are given the types excluded by the second rule; in such instances parentage is highly improbable, as has just been explained.

THE STANDARD Hr FACTOR AND THE EIGHT Rh TYPES

As has already been mentioned, the standard Hr factor (or the factor Hr') is the only Hr factor generally available at the present time for practical use in clinical and legal medicine. According to the theory of Race and Taylor,¹⁴ and Race and associates,¹⁵ which is now well established, this factor occurs as a partial antigen in the agglutinogens determined by genes r , r^o , R'' , and R^2 and is absent from the agglutinogens determined by genes R' and R^1 (and R^r and R^2). Briefly, therefore, standard Hr is related to the Rh' factor in the same way that M is related to N, and, to indicate this, the standard Hr factor was designated Hr' by mc.⁵

TABLE IV. CLASSIFICATION OF Rh BLOOD TYPES AND SUBTYPES

Rh BLOOD TYPES	REACTIONS WITH SERA			GENO- TYPES	REACTIONS WITH SERA		Rh SUB- TYPES	APPROXI- MATE DIS- TRIBUTION (PER CENT AMONG CAU- CASIANS IN NEW YORK)
	ANTI-Rh'	ANTI-Rh''	ANTI-Rh _o		ANTI-Hr'	ANTI-Hr''		
rh	Neg.	Neg.	Neg.	rr	Pos.	Pos.	----	13.0
Rh'	Pos.	Neg.	Neg.	$\left\{ \begin{array}{l} R'R' \\ R'r \end{array} \right\}$	Neg. Pos.	Pos. Pos.	Rh'Rh' Rh'rh	.01 1.0 } 1.0
Rh''	Neg.	Pos.	Neg.	$\left\{ \begin{array}{l} R''R'' \\ R''r \end{array} \right\}$	Pos. Pos.	Neg. Pos.	Rh''Rh'' Rh''rh	.005 0.5 } 0.5
Rh'Rh''	Pos.	Pos.	Neg.	$R'R''$	Pos.	Pos.	----	.01
rh _o	Neg.	Neg.	Pos.	$\left\{ \begin{array}{l} r^o r^o \\ r^o r \end{array} \right\}$	Pos.	Pos.	----	2.0
Rh ₁ (Rh' _o)	Pos.	Neg.	Pos.	$\left\{ \begin{array}{l} R^1 R^1 \\ R^1 R' \\ R^1 r \end{array} \right\}$	Neg.	Pos.	Rh ₁ Rh ₁	20.0
				$\left\{ \begin{array}{l} R^1 R^o \\ R^1 r^o \end{array} \right\}$	Pos.	Pos.	Rh ₁ rh	34.0 } 54.0
Rh ₂ (Rh'' _o)	Neg.	Pos.	Pos.	$\left\{ \begin{array}{l} R^2 R^2 \\ R^2 R'' \\ R^2 r \end{array} \right\}$	Pos.	Neg.	Rh ₂ Rh ₂	3.0
				$\left\{ \begin{array}{l} R^2 r^o \\ R'' r^o \end{array} \right\}$	Pos.	Pos.	Rh ₂ rh	12.0 } 15.0
Rh ₁ Rh ₂	Pos.	Pos.	Pos.	$\left\{ \begin{array}{l} R^1 R^2 \\ R^1 R^2 \\ R^1 R'' \end{array} \right\}$	Pos.	Pos.	----	14.5

As shown in Table IV, with the aid of anti-Hr' serum, the types Rh₁ and Rh' can each be divided into two subtypes, thus increasing the number of identifiable types of blood from eight to ten. In a similar manner, with the aid of anti-Hr'' serum, it will be possible to subdivide types Rh₂ and Rh'', further increasing the total number of Rh types to twelve. The designations of the subtypes were selected to indicate the most common genotype occurring among individuals belonging to the subtype in question. Again the designations have been made as simple as possible, and there is still no danger of ambiguity, because, for example, the designations of phenotypes Rh₁Rh₁ and Rh₁rh could hardly be confused with the designations of genotypes $R^1 R^1$ and $R^1 r$.

TABLE V. EXCLUSIONS OF PARENTAGE POSSIBLE WITH AID OF STANDARD ANTI-HR SERA*

MATING	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE
rh × Rh'Rh'	Rh'rh	rh, Rh'Rh' (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , rh ₀ , Rh'', and Rh'Rh'')	
rh × Rh'rh	Rh'rh and rh	Rh'Rh' (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , rh ₀ , Rh'', and Rh'Rh'')	
rh × Rh'Rh''	Rh'rh and Rh''	Rh'Rh' (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , and rh ₀)	(Rh'Rh'' and rh)
rh × Rh ₁ Rh ₁	Rh ₁ rh and Rh'rh	Rh ₁ Rh ₁ , Rh'Rh', rh ₀ , rh (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
rh × Rh ₁ rh	Rh ₁ rh, Rh'rh, rh ₀ , and rh	Rh ₁ Rh ₁ , Rh'Rh' (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
rh × Rh ₁ Rh ₂	Rh ₁ rh, Rh'rh, Rh ₂ , and Rh''	Rh ₁ Rh ₁ , Rh'Rh'	(Rh ₁ Rh ₂ , Rh'Rh'', rh ₀ , and rh)
Rh'Rh' × Rh'Rh'	Rh'Rh'	Rh'rh, rh (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , rh ₀ , Rh'', and Rh'Rh'')	
Rh'Rh' × Rh'rh	Rh'Rh' and Rh'rh	rh (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , rh ₀ , Rh'', and Rh'Rh'')	
Rh'rh × Rh'rh	Rh'Rh', Rh'rh, and rh	(Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , rh ₀ , Rh'', and Rh'Rh'')	
Rh'Rh' × Rh''	Rh'rh and Rh'Rh''	Rh'Rh', Rh'', rh (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , and rh ₀)	
Rh'rh × Rh''	Rh'rh, Rh'Rh'', Rh'', and rh	Rh'Rh' (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , and rh ₀)	
Rh'Rh' × Rh'Rh''	Rh'Rh' and Rh'Rh''	Rh'rh, Rh'' (Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , and rh ₀)	(rh)
Rh'rh × Rh'Rh''	Rh'Rh', Rh'rh, Rh'', and Rh'Rh''	(Rh ₁ , Rh ₂ , Rh ₃ , Rh ₄ , and rh ₀)	(rh)
Rh'Rh' × rh ₀	Rh ₁ rh and Rh'rh	Rh ₁ Rh ₁ , Rh'Rh', rh ₀ , rh (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'rh × rh ₀	Rh ₁ rh, Rh'rh, rh ₀ , and rh	Rh ₁ Rh ₁ , Rh'Rh' (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'Rh' × Rh ₁ Rh ₁	Rh ₁ Rh ₁ , and Rh'Rh'	Rh ₁ rh, Rh'rh, rh ₀ , rh (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'Rh' × Rh ₁ rh	Rh ₁ Rh ₁ , Rh ₁ rh, Rh'Rh', and Rh'rh	rh ₀ , rh (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'rh × Rh ₁ Rh ₁	Rh ₁ Rh ₁ , Rh ₁ rh, Rh'Rh', and Rh'rh	rh ₀ , rh (Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'rh × Rh ₁ rh	Rh ₁ Rh ₁ , Rh'Rh', Rh ₁ rh, Rh'rh, rh ₀ , and rh	(Rh ₂ , Rh'', Rh ₃ , Rh ₄ , and Rh'Rh'')	
Rh'Rh' × Rh ₂	Rh ₁ rh, Rh'rh, Rh ₂ , Rh ₃ , and Rh'Rh''	Rh ₂ , Rh'', rh ₀ , rh, Rh ₁ Rh ₁ , and Rh'Rh'	
Rh'rh × Rh ₂	Rh ₁ rh, Rh'rh, Rh ₂ , Rh ₃ , Rh'Rh'', Rh ₂ , Rh'', rh ₀ , and rh	Rh ₁ Rh ₁ , and Rh'Rh'	
Rh'Rh' × Rh ₁ Rh ₂	Rh ₁ Rh ₁ , Rh'Rh', Rh ₁ Rh ₂ , and Rh'Rh''	Rh ₂ , Rh'', rh ₀ , and rh	Rh ₁ rh and Rh'rh

*Types enclosed in parentheses are those excluded under the theory of six standard genes, without the aid of anti-Hr serum (see Table III).

TABLE V—CONT'D

MATING	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS POSSIBLE	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS EXCLUDED	IF CHILD BELONGS TO ONE OF FOLLOWING TYPES, PARENTAGE IS HIGHLY IMPROBABLE
Rh'rh × Rh,Rh ₂	Rh,Rh ₁ , Rh,rh, Rh'Rh', Rh'rh, Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh''		(rh ₀ and rh)
Rh'' × Rh'Rh''	Rh'rh, Rh'', and Rh'Rh''	Rh'Rh' (Rh ₁ , Rh ₂ , Rh,Rh ₂ , and rh ₀)	(rh)
Rh'' × Rh,Rh ₁	Rh,Rh ₂ , Rh,rh, Rh'Rh'', and Rh'rh	Rh,Rh ₁ , Rh'Rh', Rh ₂ , Rh'', rh ₀ , and rh	
Rh'' × Rh,rh	Rh,Rh ₂ , Rh,rh, Rh'Rh'', Rh'rh, Rh ₂ , Rh'', rh ₀ , and rh	Rh,Rh ₁ , and Rh'Rh'	
Rh'' × Rh,Rh ₂	Rh,Rh ₁ , Rh'Rh'', Rh'rh, Rh ₂ , and Rh''	Rh,Rh ₁ , and Rh'Rh'	(rh ₀ and rh)
Rh'Rh'' × Rh'Rh''	Rh'Rh', Rh'', and Rh'Rh''	(Rh ₁ , Rh ₂ , Rh,Rh ₂ , and rh ₀)	Rh'rh (rh)
Rh'Rh'' × rh ₀	Rh,rh, Rh'rh, Rh ₂ , and Rh''	Rh,Rh ₁ and Rh'Rh'	(Rh,Rh ₂ , Rh'Rh'', rh ₀ , and rh)
Rh'Rh'' × Rh,Rh ₁	Rh,Rh ₁ , Rh'Rh', Rh,Rh ₂ , and Rh'Rh''	Rh ₂ , Rh'', rh ₀ , and rh	Rh,rh, Rh'rh
Rh'Rh'' × Rh,rh	Rh,Rh ₁ , Rh'rh, Rh'Rh', Rh'rh, Rh,Rh ₂ , Rh'Rh'', Rh ₂ , and Rh''		(rh ₀ and rh)
Rh'Rh'' × Rh,Rh ₂	Rh,Rh ₁ , Rh'Rh', Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh''		Rh,rh, Rh'rh (rh ₀ and rh)
rh ₀ × Rh,Rh ₁	Rh,rh and Rh'rh	Rh,Rh ₁ , Rh'Rh', rh ₀ , rh (Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh'')	
rh ₀ × Rh,rh	Rh,rh, Rh'rh, rh ₀ , and rh	Rh,Rh ₁ , Rh'Rh' (Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh'')	
rh ₀ × Rh,Rh ₂	Rh,rh, Rh'rh, Rh ₂ , and Rh''	Rh,Rh ₁ and Rh'Rh'	(Rh,Rh ₂ , Rh'Rh'', rh ₀ , and rh)
Rh,Rh ₁ × Rh,Rh ₁	Rh,Rh ₁ and Rh'Rh'	Rh,rh, Rh'rh, rh ₀ , rh (Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh'')	
Rh,Rh ₁ × Rh,rh	Rh,Rh ₁ , Rh,rh, Rh'Rh', Rh'rh, rh ₀ , and rh	rh ₀ , rh (Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh'')	
Rh,Rh ₁ × Rh ₂	Rh,rh, Rh,Rh ₂ , Rh'rh, and Rh'Rh''	(Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh'')	
Rh,rh × Rh ₂	Rh,rh, Rh,Rh ₂ , Rh'rh, Rh'Rh'', Rh ₂ , Rh'', rh ₀ , and rh	Rh,Rh ₁ , Rh'Rh', Rh ₂ , Rh'', rh ₀ , and rh	
Rh,Rh ₁ × Rh,Rh ₂	Rh,Rh ₁ , Rh'Rh', Rh,Rh ₂ , and Rh'Rh''		Rh,rh and Rh'rh
Rh,rh × Rh,Rh ₂	Rh,Rh ₁ , Rh,rh, Rh'Rh', Rh'rh, Rh,Rh ₂ , Rh'Rh'', Rh ₂ , and Rh''		(rh ₀ and rh)
Rh ₂ × Rh,Rh ₂	Rh,rh, Rh'rh, Rh ₂ , Rh'', Rh,Rh ₂ , and Rh'Rh''	Rh,Rh ₁ and Rh'Rh'	(rh ₀ and rh)
Rh,Rh ₂ × Rh,Rh ₂	Rh,Rh ₁ , Rh'Rh', Rh,Rh ₂ , Rh'Rh'', Rh ₂ , and Rh''		Rh,rh, Rh'rh (rh ₀ and rh)

The theory of Race and Taylor leads to the following two additional rules of heredity, which are of practical value in problems of disputed parentage: (1) The factor Ii_r' cannot appear in the blood of a child unless present in the blood of one or both parents. (2) Parents of type Rh_1Rh_1 or $Rh'Rh'$ cannot have children of type rh , rh_0 , Rh'' , or Rh_2 . Similarly, parents of type rh , rh_0 , Rh'' , or Rh_2 cannot have children of type Rh_1Rh_1 or $Rh'Rh'$.

In a study of the Ii_r factor and Rh blood types in a series of eighty-one families with 127 children, Sonn, Polivka, and I have not encountered a single contradiction of these two laws of heredity.¹⁰ Similar results have been obtained by Race and associates^{16, 17} in studies on a larger series of families. Moreover, investigations on the distribution of the Ii_r factor and Rh blood types in the general population have also yielded results in conformity with the theoretical expectations.^{4, 5} Therefore, it seems justified to apply these two laws without qualification in cases of disputed parentage. In fact, an exclusion of parentage based on the Rh- Ii_r tests has already been accepted by at least one court as the principal basis for the acquittal of a falsely accused man in a paternity proceeding.

The practical conclusions that can be drawn from the two laws of heredity when applying the Ii_r -Rh tests in cases of disputed parentage are presented in Table V. By increasing the number of identifiable Rh blood types from eight to ten, the use of anti- Ii_r serum has considerably increased the number of possible matings which must be included in such a table. We have omitted from Table V those matings, such as $rh \times rh$ and $rh \times Rh''$, which are given in Table III and in which the use of standard anti- Ii_r serum does not affect the possibilities. In the remaining matings listed in Table V, those types which are excluded among the children on the basis of the six-gene theory without the aid of anti- Ii_r serum have been placed in parentheses, in order to separate them from the types excluded on the basis of the Rh- Ii_r theory just discussed.

SUMMARY

Tables I to V have been presented in order to facilitate the application of the Rh blood types and standard Ii_r factor in cases of disputed parentage.

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THE RELATION OF PHYSIOLOGIC VARIABLES TO CIRCULATION TIME

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CIRCULATION times reported in the literature on presumably normal subjects exhibit very wide variations. Bernstein and Simkins,¹ using magnesium sulfate, found a range of from 7 to 18 seconds. Kvale and Allen,² using a "calcium and magnesium mixture," found a range of from 5 to 24 seconds, with an average of 13.7 seconds. Smith, Allen, and Craig,³ using sodium cyanide, reported a range of from 12.4 to 33 seconds, with an average of 20 seconds. Such wide ranges in normal values make it obvious that one could attach significance to a measurement of circulation time only when the value obtained differs very widely from the average. The measurements reported here were made in an attempt to find some other easily measured factor or factors having a definite correlation with the circulation time, with the idea that such a finding would make it possible to narrow the range of circulation time for any specific subject and thus give added significance to the test.

METHODS

All measurements were done under basal conditions on fifty healthy medical students who understood the nature of the tests and cooperated freely. In addition to circulation time, determinations were made on (1) basal metabolic rate, (2) pulse rate, (3) respiratory rate, (4) tidal air volume, (5) vital capacity, (6) arterial blood pressure, (7) pulse pressure, (8) breath-holding time, (9) minute respiratory volume, and (10) body surface area.

The circulation time was determined by using aminophylline‡ as the reagent, as was suggested by Koster and Sarnoff,⁴ with the exception that 0.5 ml. containing 120 mg. of aminophylline was used rather than 1.0 ml. containing 240 mg. as they suggested. The time recorded as circulation time was from the beginning of the injection to the reaction. The reaction, or end point, is purely objective and can easily be taken before the subjective reaction occurs. The end point is usually a sharp inspiration (prolonged and deep) which may be initiated at any phase of the respiratory cycle. When grimaces or swallows preceded the inspirations, they were used as end points. Such reactions were uncommon.

The basal metabolic rate was determined by using the Benedict-Roth apparatus, the only deviation from the accepted methods being that the students

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were not confined to the building for the night preceding the test morning but were allowed to present themselves at the laboratory after having carried out the other accepted precautions.

The respiratory minute volume was calculated from the tracing obtained in measuring the basal metabolic rate, using an interval of from 3 to 6 minutes, depending upon the regularity of the tracing.

The breath-holding time was measured by giving the subject a stop watch and instructions to determine how long he could hold his breath. There was no period of previous hyperventilation, and the subject was entirely alone and therefore not on a competitive basis. The test was usually terminated when the subject experienced a moderate discomfort.

The other measurements were carried out by accepted standard procedures.

In order to standardize the results as much as possible, each of us performed the same specific duty for each measurement.

RESULTS

The results obtained are summarized in Table I which shows the range, the mean, and the correlation coefficient of each of the tests with circulation time.

Examination of Table I shows that the correlation coefficient between the pulse rate and circulation time is -0.52 , which indicates an inverse relation between these values. This value of the correlation coefficient is well above the 1 per cent level and is therefore significant.² The correlation coefficient between breath-holding time and circulation time is $+0.33$, which indicates a probably significant direct correlation.

All of the other measurements, including that of the body surface area in square meters, the correlation coefficient of which is not given, failed to show any significant correlation with circulation time.

TABLE I. VALUES FOR PHYSIOLOGIC VARIABLES IN FIFTY NORMAL YOUNG ADULTS AND CORRELATION COEFFICIENTS FOR EACH WITH CIRCULATION TIME

	LOW	HIGH	MEAN	CORRELATION COEFFICIENT
Circulation time	5.5	32.8	16.0	
Reclining pulse rate	44.0	100.0	71.0	-0.52
Basal metabolic rate	70.0	122.0	92.6	-0.2061
Room temperature ($^{\circ}$ C.)	21.0	29.0	24.7	-0.184
Oral temperature ($^{\circ}$ F.)	97.6	100.0	98.57	-0.144
Minute volume (Liters)	3.25	11.81	5.46	-0.100
Vital capacity (Milliliters)	2678.0	6556.0	4283.0	-0.059
Breath-holding time (Seconds)	33.0	167.0	69.0	$+0.333$
Reclining respiratory rate	8.0	20.0	14.0	$+0.0019$
Standing respiratory rate	8.0	22.0	16.0	-0.0064
Reclining diastolic blood pressure	56.0	90.0	76.6	-0.2601
Reclining systolic blood pressure	98.0	140.0	117.4	-0.1880
Reclining pulse pressure	20.0	64.0	40.0	-0.0165
Standing diastolic blood pressure	72.0	100.0	87.9	-0.1950
Standing systolic blood pressure	104.0	148.0	121.4	-0.2670
Standing pulse pressure	16.0	64.0	33.3	-0.1060
Distance from cubital fossa to sternal notch (Cm.)	36.0	50.0	43.7	$+0.0136$

DISCUSSION

As is indicated in Table I, we observed in young healthy adults a circulation time which averaged 16 seconds with a range of from 5.5 to 32.8 seconds. Using the same reagent, Koster and Sarnoff⁴ observed a range of from 7.1 to 20.4, with a mean of 12.4 seconds. They used as subjects postoperative patients with no cardiovascular disease. Bellis, Doss, and Graft⁶ observed a decreased circulation time following operation. This they attributed to increased metabolic demands of the organism, which is manifested by increased temperature, pulse rate, and respiratory rate. Smith and Allen⁷ also reported that circulation time is prolonged after the fourth postoperative day but is usually decreased during the first twenty-four to forty-eight hours. Feinsilver⁸ found the circulation time decreased following chest operations when the patient was given sufficient time to adjust to the new condition. The observations of these various workers concerning postoperative patients may account for the difference of our mean as compared with that of Koster and Sarnoff.⁴

The following observations with regard to pulse rate and its relation to circulation time, though not treated statistically by the various workers, seem to substantiate our results. Averbuck and Friedman⁹ attribute the decreased circulation time in children to the rapid heart rate and/or the short distance, but the latter in our experience shows no correlation. Maey, Claiborne, and Hurxthal,¹⁰ using dechalin in normal subjects, observed an average circulation time of 13.0 seconds, but the pulse rate averaged 87, which is 16.0 beats per minute higher than we observed, with an average circulation time of 16.0 seconds. Kvale and Allen¹¹ observed that atropine-induced tachycardia in human subjects, with little change in blood pressure or skin temperature, produced a decrease in circulation time.

To our knowledge, no attempt at correlation of circulation time to breath-holding time has been reported previously. This correlation in itself may be of no significance but is mentioned as an interesting observation. Though there seems to be a significant direct relationship between circulation time and breath-holding time, this is applicable only in the normal subject, as it is a well-known fact that patients with dyspnea of cardiac origin are obviously short of breath but have prolonged circulation times.

Attention is called to the average basal metabolic rate of 92.6 per cent. Although this value is definitely lower than the usually accepted mean, such a figure has been repeatedly shown to be compatible with this geographic area. Inasmuch as it would seem logical to assume that the metabolic demands would be reflected in the cardiac output and hence in the circulation time, we were somewhat surprised to find that basal metabolic rate showed no correlation with circulation time. However, a review of the literature fully supports our findings. Maey, Claiborne, and Hurxthal¹⁰ observed that circulation time was increased in hypothyroidism but not in hypometabolic states without myxedema and not in hypopituitarism. Baer and Isard¹² found that circulation time could be correlated with basal metabolic rate in hypothyroid and hyperthyroid states,

but they found no such correlation in other glandular diseases, in postnephrosal hypertension, or in normal subjects. Although they did not conclude this, these facts are noted if their graphs are carefully studied. Goldberg,¹² Kvale, and Allen² have shown that there is almost an absolute inverse relation of circulation time to basal metabolic rate in hyperthyroidism. There seems to be, therefore, some specific effect of the thyroid states on circulation time as compared with other metabolic disturbances and with the normal. This is further borne out by Boothby and Ryncarson¹⁴ who noted that the cardiac output is greater in hyperthyroidism with a given oxygen consumption than in a normal subject exercised to bring his oxygen consumption to the same level.

The other physiologic variables listed in Table I show no significant correlation with circulation time.

SUMMARY AND CONCLUSIONS

Circulation time with the use of aminophylline was determined in fifty healthy young adults. The mean time was 16 seconds, with a range of from 5.5 to 32.8 seconds.

At the same time on the same subjects, the following measurements were made: reclining pulse rate, basal metabolic rate, oral temperature, minute respiratory volume, vital capacity, breath-holding time, respiratory rate, reclining and standing systolic, diastolic, and pulse pressures, and distance from cubital fossa to sternal notch.

Correlation coefficients between each of these measurements and circulation time were determined, revealing a significant inverse correlation between pulse rate and circulation time and a probably significant direct correlation between circulation time and breath-holding time. No other correlations were significant.

No untoward reactions to aminophylline in the dosage used were encountered.

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LABORATORY METHODS

A PRIMARY STANDARD FOR THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN

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THE methods in general clinical use for the determination of hemoglobin fall into two categories: the older visual methods in which the color of the blood preparation obtained is compared with a colored standard and the photoelectric methods in which the light absorption of the solution is measured by means of a colorimeter which has previously been calibrated, usually by the manufacturer of the instrument. Neither of these methods has proved wholly adequate, since the colored standards may not accurately represent the color given by the blood solutions and are subject to changes as a result of aging, while the photoelectric instruments cannot be relied upon to retain their original calibration over an indefinite period of time. The previously available methods for the standardization of hemoglobin solutions, such as the determination of iron content or gas capacity, are too cumbersome for the average clinical laboratory.

Several methods based on the use of crystalline hemin as the primary standard have recently been suggested for the standardization of hemoglobin. Clegg and King¹ and King, Gilchrist, and Delory² have used solutions of crystalline hemin in N/10 NaOH as a primary standard for the determination of hemoglobin as alkaline hematin. Drabkin³ recommends hemin dicyanide prepared from hemin as a primary standard for the determination of hemoglobin as cyanmethemoglobin. While this latter method is probably the most precise of the colorimetric procedures, there is some objection to the routine use of cyanide, particularly in hospital laboratories.

In this laboratory we have employed the alkaline hematin method, with standards prepared by solution of crystalline hemin in N/10 borate buffer at pH 9.4. These standard solutions are superior to the more alkaline solutions used by King and his co-workers in that they are more stable and have absorption spectra more closely resembling the spectrum of alkaline hematin prepared from whole blood. Such solutions have a further advantage in that they will not react appreciably with glass; they can therefore be kept in sealed tubes which might be used directly in the colorimeter for checking the calibration values. The stability of dilute crystalline hemin solutions sealed into colorimeter tubes is now under investigation, and the results will be described in a later communication.

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EXPERIMENTAL

Hematin Solutions.—Alkaline hematin was prepared from human blood according to the method of Clegg and King, using oxalated blood samples standardized by means of the Beckman spectrophotometer. The blood samples were diluted 1:251 in N/10 NaOH, heated for five minutes in a boiling water bath, and immediately cooled. It has been established that under these conditions the conversion of hemoglobin to alkaline hematin is complete and reproducible.¹

Crystalline hemin was prepared from sheep blood according to the procedure of Cliffoern, Meloeke, and Elvehjem,⁴ and a commercial preparation was obtained.⁶ The iron content of these preparations was determined by a microgravimetric method and by the colorimetric method described by Delory.⁵ The results are given in Table I. Both preparations gave identical results; those reported in the following sections were obtained with the commercial preparation.

TABLE I

	Fe CONTENT	PURITY
Theoretical	8.57 per cent	
Sheep hemin, gravimetric	8.64 per cent	101 per cent
Sheep hemin, colorimetric	8.57 per cent	100 per cent
Commercial hemin, gravimetric	8.55 per cent	100 per cent
Commercial hemin, colorimetric	8.37 per cent	98 per cent

Stock solutions of crystalline hemin containing about 1 mg. per cubic centimeter were prepared by dissolving accurately weighed quantities in N/10 borate buffer at pH 9.4⁹. These solutions were allowed to age in the refrigerator and then were accurately diluted with the borate buffer to give solutions of final concentration ranging from 10 to 50 mg. per liter. The spectrophotometric and colorimetric measurements were made on the same day the dilutions were prepared.

For the spectrophotometric measurements, nine solutions diluted from four stock hemin solutions were used; for the colorimetric measurements, a total of fourteen solutions diluted from four stock solutions were prepared. The stock solutions had been aged from one to thirty days. Twelve standardized blood samples, diluted in duplicate with 1:251 blood diluting pipettes, were used in the calibration of the colorimeters.

Absorption Spectra.—The absorption spectra were determined with a Beckman spectrophotometer using 10 mm. Correx cells and an effective slit width of 40 Å. In Fig. 1 are compared the absorption spectra of the various preparations of alkaline hematin. It will be observed that alkaline hematin prepared by dissolving crystalline hemin in N/10 NaOH (Curve II) differs considerably from alkaline hematin prepared from whole blood (Curve I), particularly below 5,000 Å and between 5,500 and 6,000 Å. These differences are largely absent in the case of solutions in N/10 borate buffer at pH 9.4 (Curves III and IV).

Stability of the Crystalline Hemin Solutions.—As has been pointed out by Collier,⁷ solutions of crystalline hemin in N/10 NaOH undergo a progressive change resulting in increases in absorption in the blue and red. The spectrum

*From the Eastman Kodak Company; Rochester, N. Y.

gradually approaches that of hemin dissolved at pH 9.4, although the solutions do not become stable even after two months at room temperature. In the case of solutions buffered at pH 9.4, a small initial change is observed during the first several hours (compare Curves III and IV), but the solutions then appear to be stable. Since this preliminary change proceeds very slowly in dilute solution, stock solutions containing 1 mg. per cubic centimeter of hemin are kept overnight before they are diluted for the absorption measurement. These stock

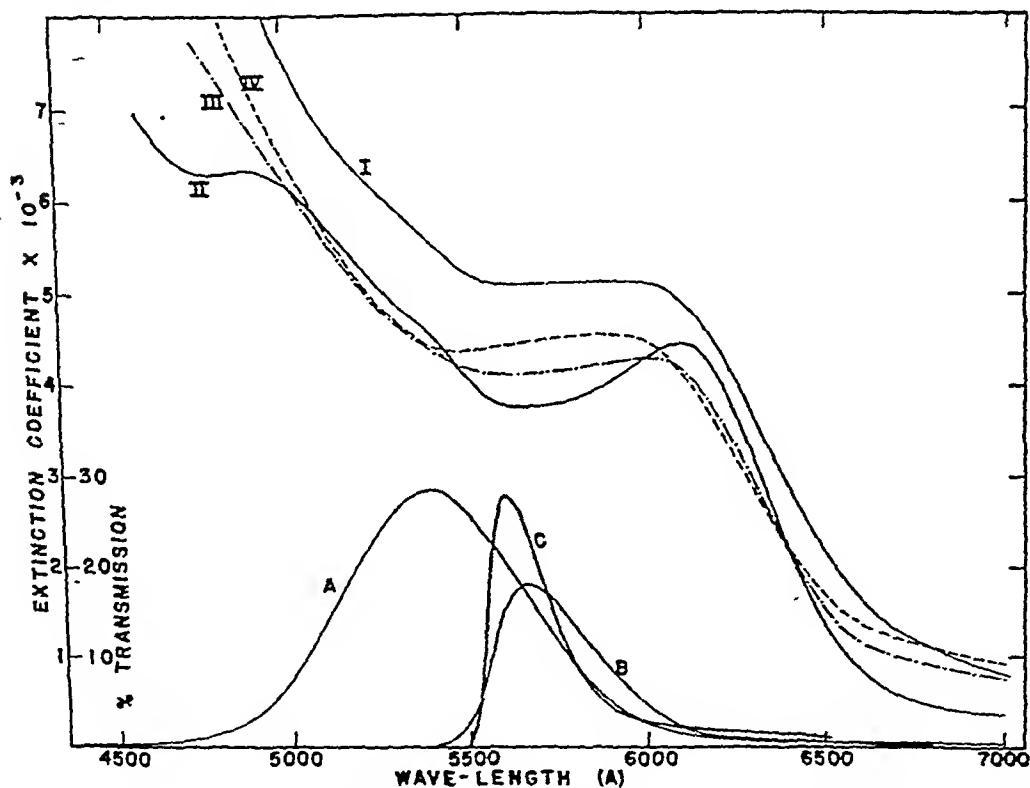


Fig. 1.—The absorption spectra of solutions of alkaline hematin. Curve I, alkaline hematin prepared from whole blood in N/10 NaOH; Curve II, crystalline hemin in N/10 NaOH, fresh solution; Curve III, crystalline hemin in N/10 borate buffer pH 9.4, fresh solution; Curve IV, crystalline hemin in N/10 borate buffer pH 9.4, diluted from aged stock solutions. Curve B represents the transmission in per cent for the 5,700 A filter described in the text. Curve A is the transmission of the Chance green filter; Curve C the transmission of the Evelyn 565 filter.

solutions may be used for one month. In the case of two preparations which had been kept five and eleven months, respectively, a decrease in absorption in the green was observed. Stock solutions should be discarded after they are one month old, and standards diluted from the stock solutions should be prepared fresh on the day they are required.

Crystalline Hemin as a Primary Standard.—The solutions of hemin in borate buffer have been applied to the analysis of blood by the alkaline hematin method, using the portable photoelectric colorimeter previously described⁸ as well as the Evelyn colorimeter. Two of the portable instruments were used in the study. For the determination of alkaline hematin, a filter was selected to

give maximum transmission at approximately 5,700 Å, as shown in Fig. 1, Curve B. This filter is composed of the following Corning glasses: No. 9780, 2.5 mm.; No. 3482, 2.7 mm.; and No. 5031, 1.5 mm. With this filter, both solutions of alkaline hematin, whether from blood or from crystalline hematin, follow the Lambert-Beer law. From theoretical considerations, the 5,700 Å filter should be superior to the Chance green filter used by Clegg and King, Curve A, since most of the light transmitted coincides with the flat portion of the alkaline hematin spectrum. With the Evelyn colorimeter, the 565 filter, Curve C was used.

In Table II are given the results obtained from a comparison of solutions of alkaline hematin prepared from crystalline hemin with solutions of alkaline hematin prepared from blood as previously described.

TABLE II. HEMOGLOBIN EQUIVALENT OF CRYSTALLINE HEMIN IN BORATE BUFFER AT PH 9.4

INSTRUMENT	BLOOD IN N/10 NaOH (C IN GM./100 C.C.)	HEMIN IN N/10 BORATE BUFFER PH 9.4 (C IN MG./LITER)	MG./LITER HEMIN EQUIVALENT TO 16.7 GM./100 C.C. HEMOGLOBIN
Colorimeter A (1)	$\log \frac{100}{T} = 0.0131C$	$\log \frac{100}{T} = 0.00729C$	30.0
Colorimeter A (2)	$\log \frac{100}{T} = 0.0121C$	$\log \frac{100}{T} = 0.00628C$	30.2
Colorimeter B (1)	$\log \frac{100}{T} = 0.0133C$	$\log \frac{100}{T} = 0.00748C$	29.7
Colorimeter B (2)	$\log \frac{100}{T} = 0.0130C$	$\log \frac{100}{T} = 0.00746C$	29.1
Evelyn	$\log \frac{100}{T} = 0.217C$	$\log \frac{100}{T} = 0.01250C$	29.0
		Average	29.6

For each colorimeter, a calibration curve was constructed by plotting the logarithm of the transmission against the concentration of hemoglobin in the blood sample. A similar plot was made for the crystalline hemin data. Each of the curves was fitted by the method of least squares. In Table II are contained the equations obtained and the values of the hemoglobin equivalent for the hemin standards, computed by simultaneous solution of the equation pairs.

The calibrations designated (1) and (2) for colorimeters A and B were separated by a period of a month, during which time changes in the amplifier circuit of colorimeter A were made. As will be noted from the corresponding equations, the transmission values were considerably different, yet there was no change in the value of the hemoglobin equivalent of the hemin standards. Since the amplifier changes resulted in a higher sensitivity, and therefore a lower lamp temperature, this observation is offered as an indication that such changes will not affect the value of the hemin standards.

From Table II it will be seen that 29.6 mg. of hemin per liter of N/10 borate buffer will, when prepared as described, give the same transmission as a blood sample containing 16.7 Gm. per 100 c.c. of hemoglobin when the blood samples are converted to alkaline hematin at a dilution of 1:251. In terms of molecular

concentration, 1.14 molecules of crystalline hemin are required to give the same absorption as will be obtained from 1.00 molecules of hemoglobin. This ratio is in excellent agreement with that observed spectrophotometrically at about 5,700 Å.

Presumably because of the calcium oxalate formed, oxalated blood samples give a somewhat lower transmission than do nonoxalated samples. The results given in Table II were obtained with oxalated blood. With blood diluted directly from a finger puncture, the absorption values, and therefore the color ratio, are about 3 per cent lower.

TABLE III. COMPARISON OF COLORIMETRIC ALKALINE HEMATIN AND SPECTROPHOTOMETRIC OXYHEMOGLOBIN METHODS OF HEMOGLOBIN DETERMINATION

BLOOD SAMPLE	SPECTROPHOTOMETRIC GM./100 C.C.	COLORIMETER A GM./100 C.C.	COLORIMETER B GM./100 C.C.	EVELYN GM./100 C.C.
1	13.4	13.5	13.6	13.7
2	15.5	15.7	15.5	15.3
3	17.5	18.0	17.3	18.0
4	13.4	13.5	13.4	13.7
5	13.0	13.1	13.1	13.0
6	16.3	16.3	16.2	16.2

In Table III are given the results obtained by the alkaline hematin method when compared with analyses on the Beckman spectrophotometer. The samples were determined as oxyhemoglobin at four wave lengths to insure the absence of significant quantities of other forms of hemoglobin. The spectrophotometric method had previously been calibrated by Van Slyke oxygen capacity determinations⁹ and found to agree within 1.0 per cent with this method. For the alkaline hematin method, the accuracy is about 0.2 Gm. per 100 c.c.

DISCUSSION

For the purposes of photoelectric colorimetry, standard solutions must have absorption spectra closely approximating those of the unknown solutions. Only when this condition is satisfied will changes in filter transmission, in photocell sensitivity, and in lamp color temperature affect both standard and unknown in a parallel manner and permit the standard to retain its validity in the event of such changes. It is further desirable that the standard solutions be stable over long periods of time in order to obviate the necessity for frequent preparation of fresh standards. Neither of these requirements is adequately met by solutions of crystalline hemin in N/10 NaOH. As pointed out by Collier, and in the present work, these solutions are unstable and qualitatively different in spectrum from alkaline hematin prepared from whole blood. Both the stability and similarity to blood alkaline hematin are substantially improved by solution at the lower pH.

SUMMARY

1. Standard solutions for the colorimetric determination of hemoglobin by the alkaline hematin method may be prepared from crystalline hemin in N/10 borate buffer at pH 9.4. Stock solutions containing 1 mg. per cubic centimeter must be aged overnight before dilution.

2. These standard solutions give absorption spectra similar to that given by alkaline hematin derived from blood.

3. With a yellow-green filter, 29.6 mg. crystalline hemin per liter of N/10 borate buffer at pH 9.4 will have an absorption equal to that of a blood sample containing 16.7 Gm. per 100 c.c., diluted 1:251 in N/10 sodium hydroxide. On a molecular basis, the ratio of absorption intensities is 1:1.14.

4. The accuracy of the alkaline hematin method is 0.2 Gm. per 100 c.c.

The author is indebted to Dr. E. J. King, for several specimens of the Chance green filter, to Mrs. Dorothy Peterson, for valuable assistance in the spectrophotometric measurements, and to Dr. Herman Yagoda, for the gravimetric iron determinations.

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A SIMPLE ELECTROCARDIOGRAPH CONTACT ELECTRODE FOR USE WITH THE SMALLER LABORATORY ANIMAL

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EXPERIMENTAL electrocardiogram studies in relation to nutrition, as well as to other factors that will cause disturbances of cardiac function, have always had the inherent disadvantage of not having a suitable simple electrode available for use with the smaller animal. The smaller animal is usually preferable or demanded in these types of experiments for economic reasons. The usual procedure has always been to devise a suitable electrode contact for the animal to be utilized in the study at hand. This has not always been practical, since the procedure is time consuming in the period the investigator discovers what will and what will not work satisfactorily. The expense of losing what might have been valuable records should also be taken into consideration.

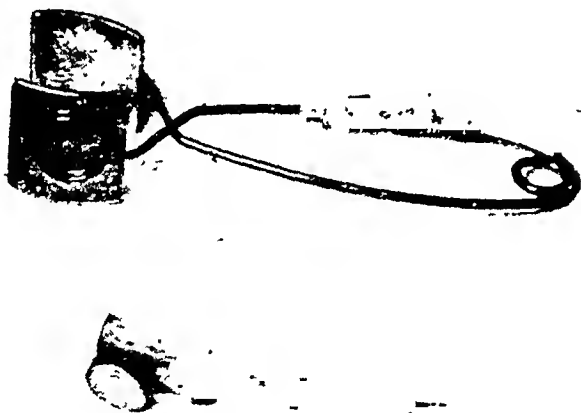


Fig. 1.—Photograph of a simple electrocardiograph electrode clip for use with smaller animals.

Illustrated in Fig. 1 is an electrode designed and constructed in this laboratory for use with the smaller unanesthetized rabbit in conjunction with dietary deficiency diseases and their effect on the heart. The electrode clip, as used with the rabbit, has a length proper of three inches, width of three-fourth inch, conventional round spring of one-half inch diameter for tension, soldered wire connection, and is insulated with a suitable material except for the inner terminal facings. Spring brass wire of 0.0575 inch diameter was used

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for constructing the clip. The tension is of the order of 125 Gm. at the electrode surface. The surface of the electrode to be attached to the animal is solid, elliptic, brass sheeting three-fourth inch long and one-half inch wide which fits snugly over the animal's leg. Materials used were of low electrical resistance. The

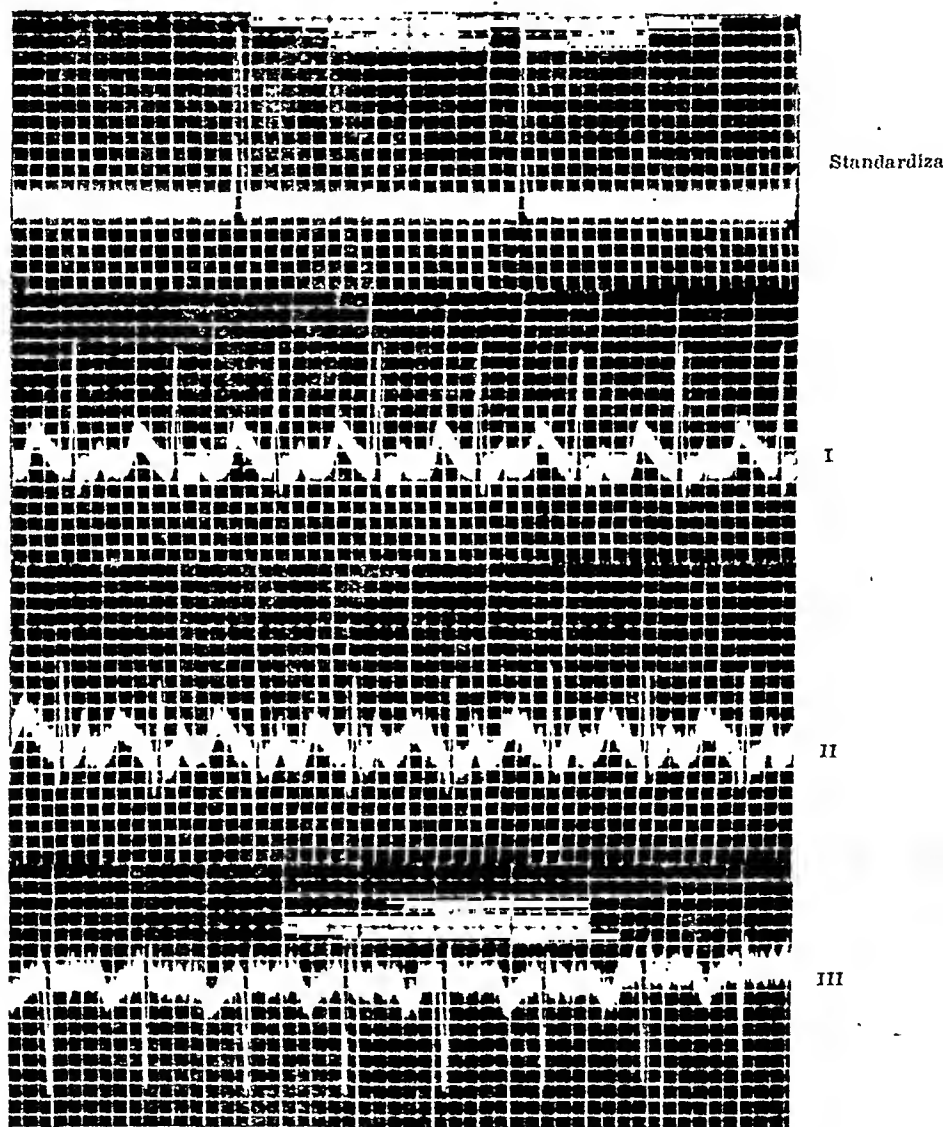


Fig. 2.—Electrocardiogram tracings of a normal rabbit weighing 1,200 grams made with the electrodes described.

length of the wire to the binding post should depend on the nature of the apparatus used to hold the animal. The design of the electrode was made of such a nature that it could be altered to be applicable for any size animal. Rabbits weighing 700 grams produced good results.

The electrode clip was designed so that the comfort of the animal could be preserved and there still be the retention of contact. It was constructed so that the pressure resulting would not stop circulation nor cause pinching of the leg and struggle from pain.

The electrocardiographic tracings of the three conventional leads of a normal rabbit, using the previously described electrode clips, are presented in Fig. 2. The animal was allowed to sit without anesthesia, and without being in a box, to prevent struggling. The clip electrodes were attached to the animal with contact being made through the use of concentrated sodium chloride as the electrolyte or by use of ECG paste. The spring held the electrode in place on the leg. If necessary, brief anesthetic quantities of pentothal sodium may be used to quiet the animal and dispel muscle action potentials without interfering with the electrocardiogram.*

The instrument used was a General Electric, Victor, Model A Electrocardiograph. The sensitivity was set at 4 on the instrument. Upward deflection represents 1 millivolt for 1 cm. displacement. The time interval, vertical lines, represent one twenty-fifth of a second per line. With this electrode clip, the ordinary electrocardiograph was found to be of use in small animal studies.

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A NEW TYPE OF GLASS CAGE FOR METABOLISM STUDIES*

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FOR metabolism experiments with small animals, cages which provide a satisfactory method for separation of fecal matter from urine are highly desirable. Most of the cages described in the literature to the present time are modifications of one type. Probably the earliest and most satisfactory of these variations was used and described by Mitchell and Carman.[‡] Cages of this type have several disadvantages resulting primarily from the use of filter paper to absorb the urine. First, between collection periods, feces and scattered feed are in contact with the moist filter paper, increasing the possibility of transfer of the substance under investigation. Second, the large area exposed by the filter paper may cause some loss or oxidative change to take place in the specific fraction of the urine. Third, absorption of the urine on filter paper necessitates subsequent extraction for recovery.

To avoid these objectionable features, we designed as a substitute the cage described in this article and have used groups of them very satisfactorily for metabolism studies with albino rats.

Referring to Fig. 1, the body of the cage was constructed from a one-gallon glass jug by cutting it off about one inch above the bottom and inserting the neck end through a nine-inch tripod on which it rests and is held in an upright position. A cover for the top of the cage was made from one-half inch mesh hardware cloth and was held in place by a suitable weight laid on top of the cover. The floor consists of a circle of this wire which was cut to fit inside of the jug and was suspended by wires from the upper edge. The height to which this false bottom is adjusted will depend upon the size of the rat and the distance to the watering tube inserted through the cover. A smaller circle of twenty-mesh nichrome wire gauze supported just above the neck of the jug formed a "tray" on which feces were collected. A rigid loop of wire attached to the tray made a convenient handle for removing the tray and its contents. The neck of the jug was fitted with a rubber stopper through which a straight glass tube was passed until it was flush with the small end of the stopper. The urine drained through this tube and was collected beneath in a 200 c.c. Erlenmeyer flask, which contained approximately 30 c.c. of 0.1 N sulfuric acid. The figure and description demonstrate the cage as used by us for a nitrogen metabolism experiment. Each day during the

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[‡]Mitchell, H. H., and Carman, G. G.: Biological Value for Maintenance and Growth of Proteins of Whole Wheat, Eggs, and Pork, J. Biol. Chem. 60: 613, 1924.

collection period, the feces were removed and preserved under alcohol containing several drops of concentrated H_2SO_4 . The neck and wire circles of the cage were rinsed with hot 0.1 N H_2SO_4 which drained into the flask. The urine in acid solution was transferred to a bottle containing several small

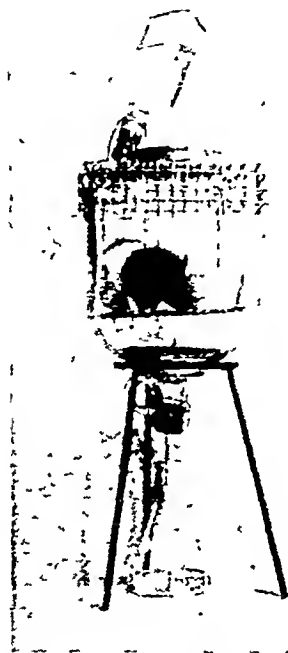


Fig. 1.—A new type of glass cage for metabolism studies.

crystals of thymol and was stored until the end of the collection period. The cage was again made habitable by flushing out the residual acid with water.

Ease of construction and efficient manipulation render these cages very desirable, especially since necessary materials are commonly found in laboratories doing biologic work with small animals.

AN ISOLATED HEART PERFUSION SYSTEM ADAPTED TO THE DETERMINATION OF NONGASEOUS METABOLITES

WITH SAMPLE DATA UPON THE ISOLATED MONKEY HEART

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AND ETHOL SHIELDS KOELLE

STUDIES of the nongaseous metabolism of the heart during the action of drugs have not often been made. When they are, heart-lung preparations of the Starling type are most frequently employed. Data obtained upon such systems inevitably include the metabolism of the lungs, which is often significant.¹ Although a system has been devised to eliminate this difficulty,² it is not a particularly convenient apparatus. Furthermore, such systems depend upon blood for the perfusion medium. While blood is admirably suited for many purposes, it does not permit ready manipulation of concentrations and varieties of components.

The system of continuous perfusion to be described is simple and readily constructed in most laboratories. A modified Locke-Ringer solution is employed as the perfusate, allowing variable concentration of any component. Meehanograms of the heart's activity are readily obtained and show little variation or depreciation over a period of many hours. Furthermore, errors due to bacterial metabolism are largely eliminated by taking advantage of the bactericidal properties of ultraviolet light. The results of the past year's work with this system have been so satisfactory that publication of the details of the method seems warranted.

APPARATUS

In Fig. 1 is shown the customary setup for such work. The water bath, 5, surrounding the weighted reservoir, 4, is maintained at 38.5° C. and contains the outflow siphon, 1, and oxygenating tube, 2; a cold finger which effectively limits evaporation is represented by 3. Not shown is the extension of 7, the return tube, which is directed into the reservoir. The outflow tube requires an outer jacket of large rubber tubing during very cold weather but is ordinarily uninsulated. An excess length of rubber tubing is provided above the long cannula, 8, to permit removal of the cannula to a lower and more convenient position for the actual cannulation. The cannula is held in a spring clamp for ready transfer. The perfusion pressure is arranged to be no more than 50 cm. of perfusate. Raising the pressure above this level results in rapid edema formation and early failure. Care must be exercised in placing a tuft of well-

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washed glass wool in the top portion of the cannula so this will be neither too tight to permit a free flow of perfusate nor too loose to be an effective filter. A beam of ultraviolet light, 6, from a microscope substage lamp containing a mercury arc bulb* is focused upon the cannula and limits bacterial growth below 100 organisms per cubic centimeter after eight hours of perfusion, compared to several millions of organisms without irradiation. No special glass

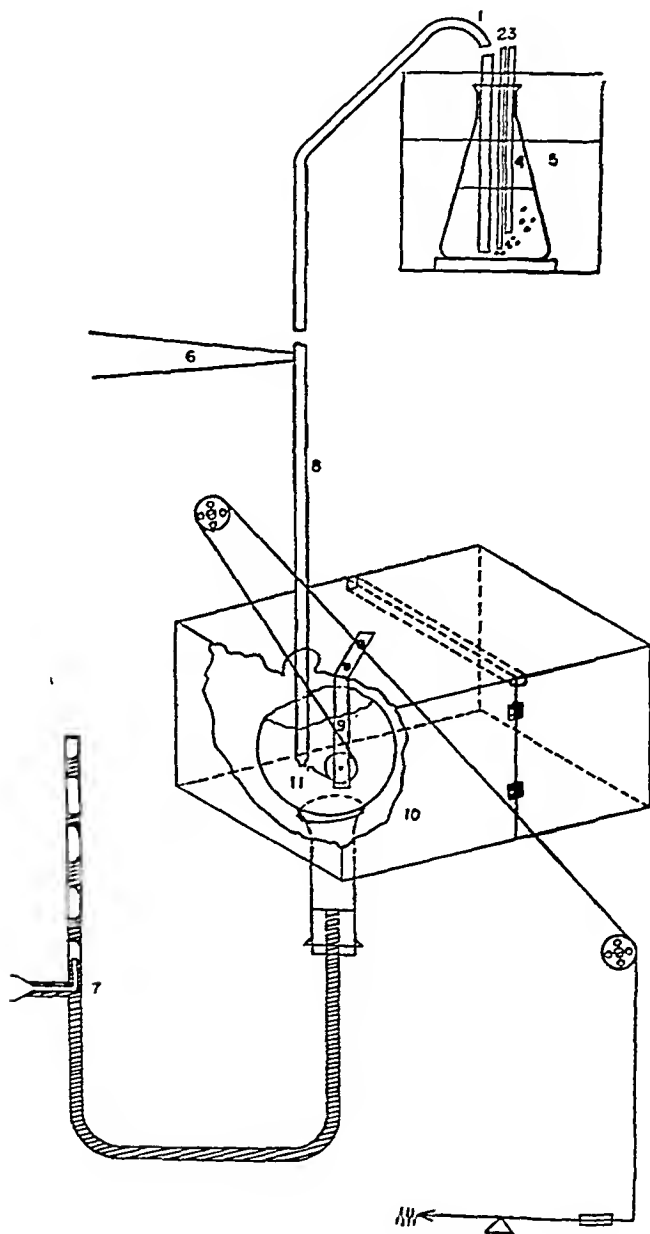
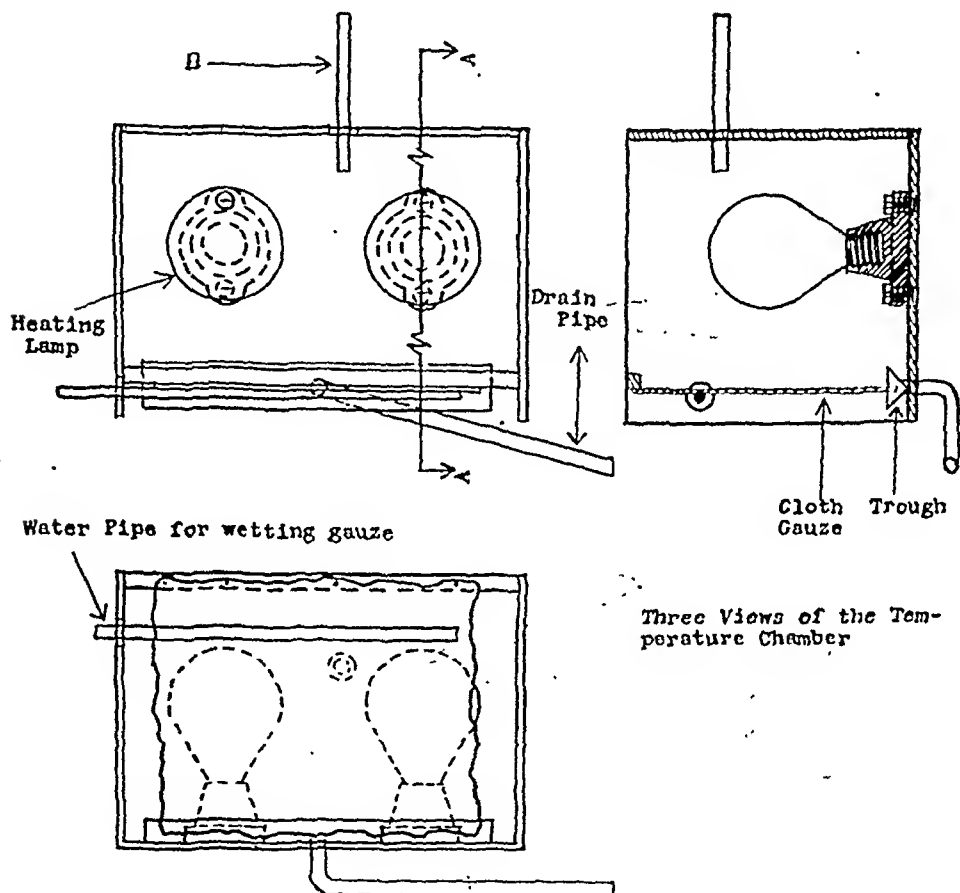


Fig. 1.—General plan view of the perfusion system. For details see text.

*General Electric Mazda AHs.

was employed for the cannula, ordinary soft glass being satisfactory. No untoward effects of the irradiation upon the experiments have been detected.

The heart chamber, 10, is maintained at 39° C. by two 150-watt electric light bulbs and a thermostat (Fig. 2). Mixing of the air within the chamber is accomplished by the air jet shown behind the bulbs. Humidity is maintained by streams of warm water sprayed upon an evaporator formed of gauze pads suspended over a drain trough placed between the bulbs and the heart.



Three Views of the Temperature Chamber

Fig. 2.—Two views of the portion of the heart chamber occupied by the heating and humidifying elements. At A are connected the terminals from the thermostat system indicated in Fig. 3. An air stream is introduced at B. Not shown are the numerous perforations along the side of the water sprayer of the humidifier.

The heart is mounted on the cannula and well within a bottomless 150 c.c. Florence flask, 11, inverted through a hole in the bottom of the chamber. This receptacle is protected by a cellulose acetate screen against splashing from the humidifying system. The necessity for sudden changes in humidity or temperature is minimized by the provision of a transparent plastic door on the heart chamber. Dipping down into the receptacle is a glass and plastic pulley, 9, mounted on a flexible lead bar attached to the top of the chamber (Fig. 3).

The lead is tightly wrapped with rubber dam to exclude accidental contamination of the perfusate. The glass bearings and axle of the pulley are simply made, the former by indenting the heated end of a glass rod with a sharp pencil and the latter by slowly turning a glass rod to a point against a fine abrasive stone. An almost frictionless bearing is thus obtained. Metal pulleys may not be used in this position, as the perfusate would soon contain toxic amounts of metallic salts.

A solid column of perfusate is maintained through the return system (Fig. 1) below the receptacle to the point, 7, where the upward air jet is admitted.

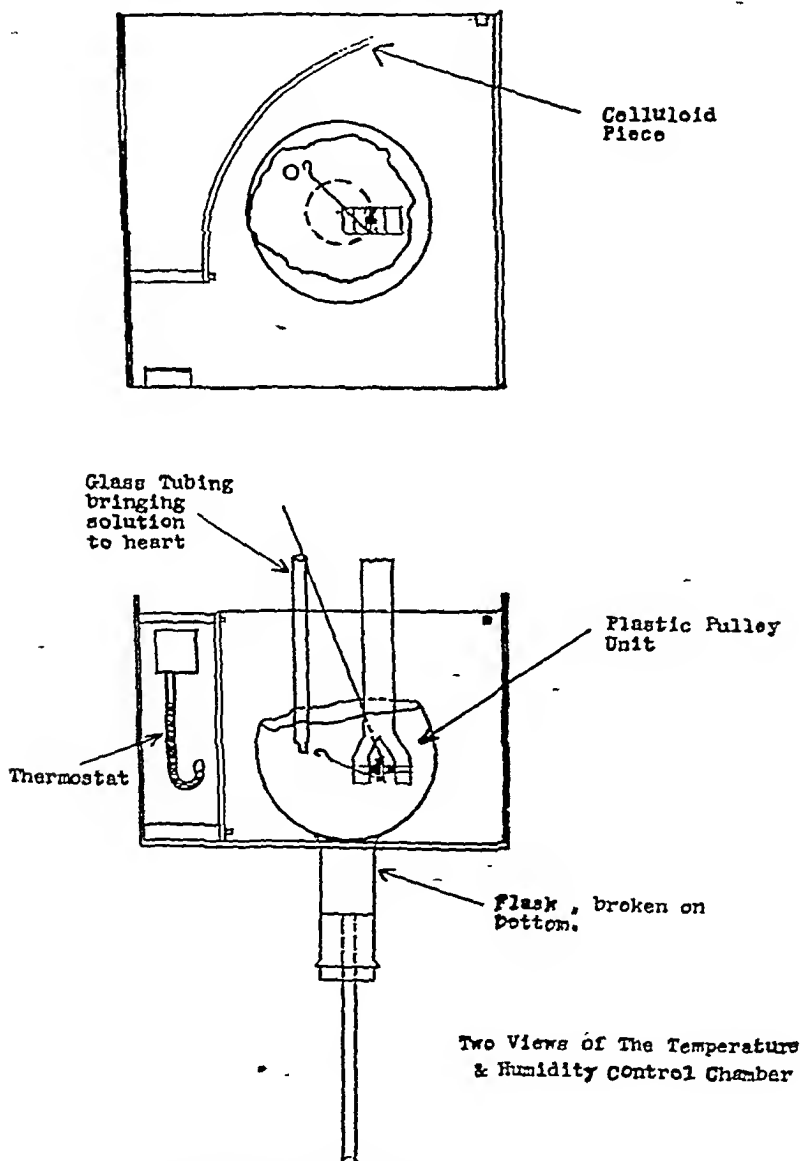


FIG. 3.—Two views of the other half of the heart chamber (see Fig. 2). The "celluloid piece" protects the heart from splashing from the humidifier.

This simple but little-used device, introduced for this purpose by Loeke and Rosenheim,³ may be said to break off portions of the perfusate column which are then blown up to the reservoir. The hydrostatic pressure of the interrupted fluid column in the upflow tube is much less than the hydrostatic pressure of the solid column in the downflow tube. A proportion of 1 cm. of solid column in the downflow tube to 3 cm. of broken column in the upflow is easily handled by the system. Some experimentation may be necessary to establish the ideal conditions for each installation.

TABLE I. COMPOSITION OF PERFUSION FLUIDS*

SUBSTRATE MATERIAL	NaCl	KCl	CaCl ₂	MgCl ₂	NaHCO ₃	SUBSTRATE
Glucose	7.0	0.42	0.24	0.20	2.1	1.80
Acetate, Na	6.0	0.42	0.24	0.20	2.1	0.77
Pyruvate, Na	6.5	0.42	0.24	0.20	1.8	0.78

*All weights are grams per liter of anhydrous salts.

The modified Loeke-Ringer solutions which have been successfully employed are indicated in Table I. All salts must be of the highest purity. Sodium chloride labelled "for biological work" (Merek) is a most suitable preparation. Other forms of chemically pure sodium chloride have often been found unsatisfactory. An anhydrous glucose manufactured by Baker Chemical Company is suitable, but ordinary U.S.P. glucose is not. A mixture of 95 per cent oxygen and 5 per cent carbon dioxide is used to oxygenate and to maintain the pH of the perfusate. The latter always measured from 7.3 to 7.4 (glass electrode) when the temperature of the reservoir was 38.5° C. All gases are admitted to the system after bubbling through distilled water.

Certain other precautions are imperative for the procurement of long-lived preparations. All water which is used to make up the perfusate is redistilled through an all-glass system, and the perfusion system is rinsed several times with this glass-distilled water after cleaning. All rubber tubing must be uncolored gum rubber and must be boiled for several hours in distilled water. The system must not be cleaned with chromate-sulfuric acid mixtures which leave significant amounts of highly toxic chromium salts despite diligent washing. Protein is removed from the glass portions of the system with hot nitric acid, but cork and rubber portions must be cleaned with soap or hydrochloric acid. Unrelaxed precaution must be observed against the introduction of metal into the apparatus where any possibility of contact with the perfusate exists. For example, condensate dripping off metal clamps is not always immediately detected, while iron filings have been found in the best glass wool. The hook used to attach the writing lever to the heart is made from a detempered stainless steel hypodermic needle. It may be pointed out that each precaution is based upon experience and cannot be overlooked successfully.

PROCEDURE

In the study for which this apparatus was employed, the procedure to be described was regularly followed. The perfusate was made up in advance,

omitting the substrate and the sodium bicarbonate. Following thorough equilibration of the solution against the gas mixture, the substrate and sodium bicarbonate were added. Failure to acidify the perfusate before addition of the bicarbonate often results in the formation of a precipitate of calcium carbonate, rendering the solution useless. A measured quantity of the perfusate was added to the system and the perfusate started through the cannula and allowed to circulate slowly until temperature, and hence pH, equilibrium was reached. The ultraviolet irradiation was begun at once. A large, flat, glass dish was placed in a convenient place to catch perfusate lost while the heart was being attached to the cannula, a screw clamp being adjusted to maintain a slow rate of flow during the process. The volume thus lost was subtracted from the amount originally in the system. The animal was killed by crushing the head, chemical anesthesia of any form having been found severely depressant to hearts obtained from such animals. The heart was quickly removed and the aorta cannulated after trimming and washing. All clots were removed by forceps and washing before mounting. Hearts which develop ventricular fibrillation during this process should be discarded even though arrest of the fibrillation is prompt, as experience has shown their inferiority. An effort was made to tie below the innominate artery without occluding the pulmonary artery, which must otherwise be cut open, occasionally inducing ventricular fibrillation. The clamp was then opened fully, the heart flushed with from 50 to 100 c.c. of fluid, the clamp tightened, and the cannula and heart were mounted in the heart chamber. The clamp was released immediately afterward. The heart lever system was securely attached to the heart and was arranged to record on a slowly moving kymograph.

Preparations thus obtained were often found to manifest little or no depreciation before nine or ten hours of perfusion, monkey hearts being more durable than rabbit hearts. Samples for analysis were removed by a syringe and a hypodermic needle from the rubber tubing above the cannula. The amount of concentration resulting from evaporation of the perfusate was corrected from Cl^- levels. Drugs may be added to the system through the top of the receptacle flask, permitting solution and distribution before entering the heart. The circulation time for added dyes was about fifty seconds in the apparatus employed.

RESULTS

In Fig. 4 are shown kymographic tracings of three control experiments on monkey hearts (*Macaca mulatta*), with a perfusate containing 0.01 M glucose. The preparations were allowed to beat for six hours, with the noteworthy minimum of failure shown. Analytic results obtained on these three hearts are presented in Table II.* The slight degree of failure (11 per cent overall) noted in Experiment 104M7 is closely paralleled by a sharp increase in the

*Glucose was determined by the method of Somogyi,⁴ pyruvic acid by the method of Friedemann and Haugen,⁵ lactic acid by the method of Barker and Summerson,⁶ and keto acids by the method of Friedemann and Haugen.⁵

production of lactic and pyruvic acids, absent in the other two experiments. The increased disappearance of glucose during the second three-hour period noted in the first two experiments is generally observed and may be interpreted as the result of the disappearance of cardiac glycogen, hearts beating in substrate-free perfusate customarily failing at the end of a three-hour period, indicating that cardiac glycogen has been exhausted. Therefore, these

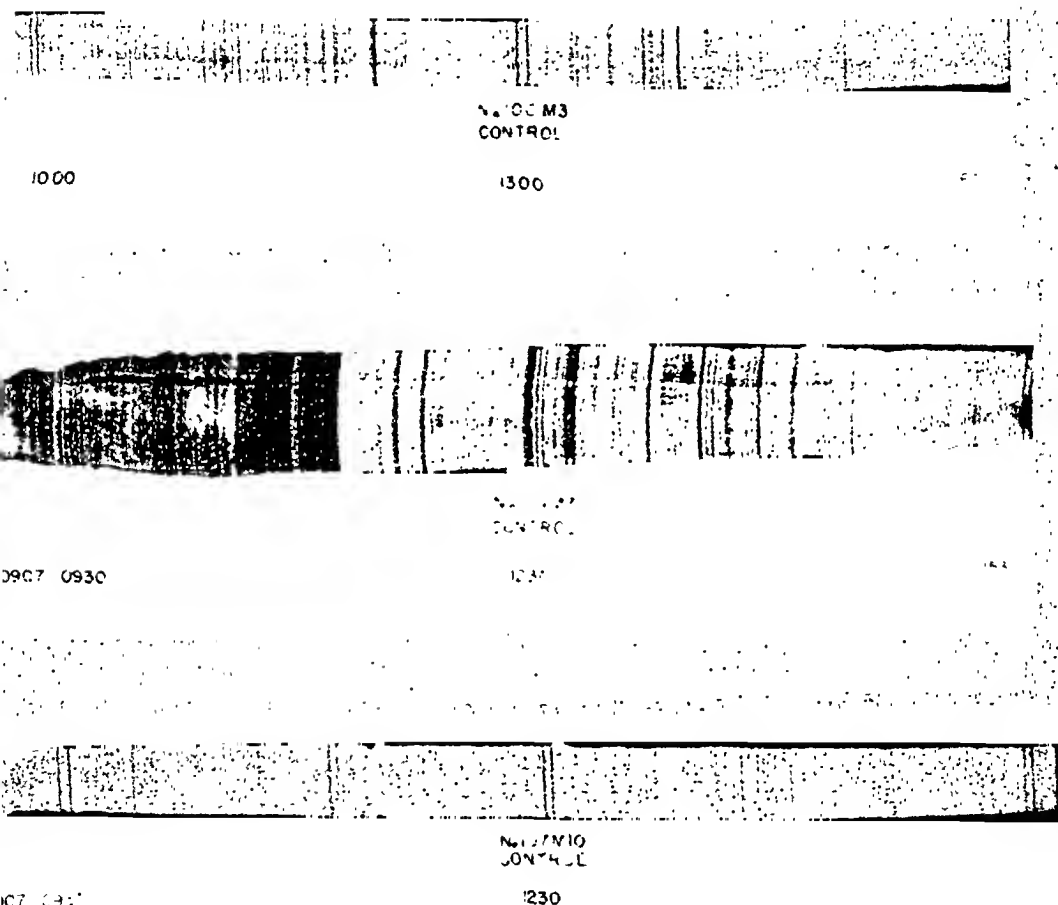


Fig. 4.—Three kymographic tracings of isolated, perfused monkey hearts (*Macaca mulatta*). Substrate 0.01 M glucose. Time is Army-Navy twenty-four hour system and represents a total perfusion time between the first and third analyses of six hours.

higher figures probably represent the actual glucose utilization of glycogen-low hearts. The absence of such a change in Experiment 107M10 is taken to imply a larger reservoir of cardiac glycogen. A sample calculation for glucose utilization (or for any other metabolite) is presented, showing corrections for evaporation and sample volume.

TABLE II. ANALYTIC DATA OBTAINED ON MONKEY HEARTS USING GLUCOSE AS SUBSTRATE IN A SERIES OF CONTROL EXPERIMENTS

EXPERIMENT	PERIOD (3-HR. INTER- VALS)	CIRCULATING LEVELS MG./DL.					UTILIZATION MG./GM. DRY WT./HR.				PER CENT OF INITIAL CONTEAC- TION
		CHLO- RIDE	GLU- COSE	LACTIC ACID	PYRU- VIC ACID	TOTAL KETO- ACIDS	GLU- COSE	LACTIC ACID	PYRU- VIC ACID	TOTAL KETO- ACID	
100M3	0	910	115	0.80	0.30	0.33	- 5.80 -21.00	-0.04 -0.80	-0.03 +0.10	-0.07 +0.15	100
	3	913	105	0.70	0.24	0.20					103
	6	929	60	2.30	0.48	0.56					109
104M7	0	906	169	1.00	0.30	0.33	- 1.40 -26.70	-0.44 +1.80	-0.07 +0.22	-0.14 +0.27	100
	3	910	168	0.50	0.22	0.17					118
	6	929	133	3.10	0.54	0.56					89
107M10	0	914	173	0.60	0.16	0.15	- 2.50 - 1.70	+0.35 +0.47	+0.10 +0.07	+0.06 +0.08	100
	3	920	172	0.90	0.24	0.20					108
	6	931	172	1.40	0.32	0.28					157

Experiment 104M7

		TIME (HR.)	MG. PER CENT (OR PER DL.)	
			GLUCOSE	CHLORIDE
Initial volume	=	700 c.c.	0	906
Initial loss	=	-50 c.c.	3	168
		650 c.c.		910
Heart weight after 24 hr. at 104° C. = 2.10 Gm.				
Sample volume = 100 c.c.				

Period I

$$\begin{array}{rcl}
 (a) & 6.50 \text{ dl.} \times 169 \text{ mg./dl.} & = 1098 \text{ mg. total} \\
 & -1.00 \text{ dl.} \times 169 \text{ mg./dl.} & = -169 \text{ mg. total} \\
 & \hline
 & 5.50 \text{ remain after sample} & 929 \text{ mg. total}
 \end{array}$$

(b) *Correction for evaporation:*

$$\begin{array}{rcl}
 & 906 \text{ mg. Cl/dl.} & \\
 & 910 \text{ mg. Cl/dl.} \times 5.50 \text{ dl.} & = 5.48 \text{ dl.} \\
 & 5.48 \text{ dl.} \times 168 \text{ mg./dl.} & = 920 \text{ mg. total} \\
 & & \hline
 & & 9 \text{ mg. used}
 \end{array}$$

$$\begin{array}{rcl}
 (c) & 9 \text{ mg./2.10 Gm. dry wt./3 hr.} & \\
 & \text{or } -1.4 \text{ mg./Gm./hr.} &
 \end{array}$$

Period II

Calculations repeated commencing with the corrected 3 hr. volume, 5.48 dl.

DISCUSSION

No data appear to be available in the literature on the behavior of isolated, perfused monkey hearts, so that no direct comparison with the data presented is possible. The data obtained, however, fall well in line with the more detailed studies on other species and have the advantage of being free from errors introduced by bacterial metabolism. The ease with which the system presented here can be routinized and adapted to many studies commends its frequent application.

SUMMARY

1. A convenient, simple, and highly satisfactory continuous perfusion system for the preparation of vigorous, long-lived, isolated hearts has been described. The system is particularly adapted for chemical analyses under bacteria-free conditions.

2. Data are presented for three experiments on the isolated, perfused monkey heart (*Macaca mulatta*), demonstrating the suitability of the method.

The authors are indebted to Lieutenant J. A. Wantuck, for the drawings of the perfusion apparatus, and to Miss Mary Rowell and Miss Geneva Luton, for much patient assistance.

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SEVERE SCURVY

A CLINICAL AND HEMATOLOGIC STUDY

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DURING the past few years we have studied nineteen patients seriously ill with scurvy, many of whom had severe anemia. This report deals primarily with the morphologic characteristics of the blood and the clinical and hematologic responses to synthetic vitamin C therapy which were observed in these subjects. Certain characteristics of the anemia which have not been described previously suggest that hemolysis may be an etiologic factor. The number of patients in this series and the severity of their disease emphasize that scurvy remains a medical and public health problem in large municipal hospitals.

MATERIAL AND METHODS

From January, 1935, to June, 1945, nineteen patients with a primary diagnosis of scurvy have been admitted to the Medical Service of the Cincinnati General Hospital.

Eleven of these patients, all of whom were admitted during 1942, 1943, 1944, and 1945, have been under our direct observation and are the subjects of the hematologic study. The eight additional patients (Table I, Cases 1 to 8) were studied only through data obtained from their hospital records. Since these eight individuals were treated in many different ways, only the hematologic observations made on admission and some of the distinctive clinical features will be reported.

On each of the eleven patients (Table II, Cases 9 to 19) detailed medical and neurologic histories were obtained and complete physical and neurologic examinations performed. Diet histories were obtained and analyzed* whenever the patients could give accurate accounts of their food habits. Red and white blood cell counts† were done on peripheral blood with pipettes and counting chambers certified by the United States Bureau of Standards. Hemoglobin was determined as oxyhemoglobin with the Evelyn photoelectric colorimeter.¹ Hematocrit determinations were made on oxalated venous blood (4 mg. potassium oxalate and 6 mg. ammonium oxalate per 5 c.c. of blood) centrifuged for thirty minutes in a Wintrobe tube at 3,000 r.p.m. Reticulocyte and platelet counts were made by the wet technique using Dameshek's method.² Cover slip preparations for cytologic study of capillary blood and sternal marrow were stained with Wright-Giemsa stains. The bone marrow specimens were obtained by sternal aspiration.

The amount of ascorbic acid in the plasma was determined in eleven patients (Cases 9 to 19) by the method of Farmer and Abt.³ Icteric indices, qualitative van den Bergh reactions, foam, iodine, and fuming nitric acid tests for bile in the urine and the dilution

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*The authors are indebted to Mrs. Vinton Siler, Miss Helen Volk, Miss Carol Jones, Mrs. Katherine Shafer Irwin, Miss Lillian Lanzl, Miss June Hamlin, and Miss Roberta Thomas, Dietetic Department, Cincinnati General Hospital, for analyses of the diets and for dietary supervision of the patients on the vitamin C restricted diet.

†All hematologic studies were performed by us assisted by Mrs. Nancy Lavery.

TABLE I. INITIAL EXAMINATION

CASE	PATIENT	SEX	AGE	YEAR	R.B.C. (MIL- LIONS)	HB. (GM.)	RETICU- LOCYTES (%)	PLATE- LETS	HE MA CRI
1	J. B.	M	75	1937	2.66	9.5	5.0	—	23
2	M. C.	M	31	1943	3.45	9.0	—	—	—
3	M. J.	F	59	1940	2.64	10.4	—	—	—
4	G. F.	M	54	1938	2.08	6.4	7.5	380,000	18
5	S. J.	M	42	1936	4.03	14.4	—	—	—
6	F. E.	M	35	1938	4.35	13.5	—	210,000	40
7	H. W.	M	32	1942	3.44	9.2	6.0	316,000	24
8	W. J.	M	16	1942	2.90	8.8	—	—	—

This data has been gathered from Cincinnati General Hospital charts.

method⁴ using Ehrlich's aldehyde reagent for urobilinogen in twenty-four hour urine specimens were performed in thirteen patients (Cases 1, 4, and 9 to 19) and were repeated in seven (Cases 11 and 14 to 19) every other day until they were normal. The amount of urobilinogen in the stool was determined for three patients (Cases 17 to 19) by Watson's method.⁵

Prothrombin times were estimated in eleven patients (Cases 4, 9, 10 and 12 to 19) by Quick's method.⁶ Total serum proteins were determined by the Kjeldahl technique in eight of the subjects (Cases 4, 10 to 13, 15, 17, and 19) and albumin-globulin ratios were determined in four (Cases 4, 10, 12 and 13). The cephalin flocculation test according to the method of Hanger⁷ and the bromsulphalein excretion test using 2 mg. dye per kilogram of body weight and the blood sample drawn thirty minutes after the injection of the dye were carried out in seven patients (Cases 11 and 14 to 19). Determinations of serum iron⁸ were performed* on three patients (Cases 17 to 19).

The diet used in this study will be called the vitamin C restricted diet. For each twenty-four hour period, it provided the following foods: a glass of boiled milk, one egg or less, cottage cheese, American cheese, polished rice, a thoroughly cooked vegetable made into purée, mashed potato, noodles, stewed dried fruit or apple sauce, syrup, crackers, jam, and coffee. The diet contained no meat, citrus fruits, tomatoes, cabbage, lettuce, or uncooked vegetables and provided about one-third of the minimum daily requirement of the B complex vitamins. It provided approximately 12 mg. of iron daily and several milligrams of vitamin C as a maximum.

Case summaries for those patients whose hematologic courses are presented in graphic form accompany their respective figures.

CLINICAL AND LABORATORY MANIFESTATIONS BEFORE THERAPY WITH VITAMIN C

Occurrence of Scurvy.—Of the nineteen patients studied, five were admitted to the Cincinnati General Hospital between 1935 and 1941, three in 1942, three in 1943, five in 1944, and three during the first six months of 1945 (see Fig. 1). The majority were admitted in the spring or early autumn months (see Fig. 2). The patients ranged in age from 16 to 84 years; twelve were older than 50 years. Eighteen subjects were men; one was a woman.

Predisposing Factors.—Thirteen of the nineteen patients were bachelors or had been widowers for more than a year. They cooked their own meals or ate alone in restaurants. In several instances, citrus fruits were too expensive for their limited budgets, but for the most part these individuals just did not

*The authors are indebted to Dr. Carl V. Moore and to Miss Virginia Minnich, M.S., Washington University, St. Louis, Mo., for the determination of these serum iron values.

PERIPHERAL BLOOD ON EIGHT PATIENTS

M.C.V.	M.C.H.	M.C.H.C.	W.B.C.	DIFFERENTIAL W.B.C.				
				SEG- MENTED NEUTRO- PHILES	LYMPHO- CYTES	MONO- CYTES	EOSINO- PHILES	BASO- PHILES
83	35	41	5,600	78	16	4	2	0
—	—	—	5,900	78	20	2	0	0
—	—	—	11,200	85	14	1	0	0
90	32	35	7,150	77	19	4	0	0
—	—	—	3,750	61	33	4	0	2
—	—	—	13,800	82	15	3	0	0
84	29	32	6,650	58	35	4	2	1
—	—	—	3,700	58	23	19	0	0

take the trouble to procure oranges, grapefruit, or tomatoes. Relatives, when there were any, were too busy in industry or in the Army or Navy to see that the elderly unproductive members of the family had adequate food.

Of the remaining six patients, scurvy developed because of the following reasons: poor dietary selection in two who were mentally deficient (Cases 2 and 6); paregoric addiction and restricted food intake in one (the only female patient, Case 3); chronic alcoholic addiction and dietary insufficiency in one (Case 12); and self-prescription of vitamin C deficient diets for dyspepsia in two (Cases 5 and 7).

In addition, the following factors were probably of some importance. Two patients had spastic hemiparesis (Cases 9 and 11), the result of old cerebrovascular accidents, and one had residual signs of Jamaica ginger paralysis (Case 17). These chronic neurologic disorders limited the ability of the subjects to obtain food. One patient had minimal active pulmonary tuberculosis (Case 2), and six had chronic bronchitis and emphysema (Cases 1, 3, 9, 11, 13, and 15). These infections may have increased their vitamin requirements.

Because of one or more of these reasons, none of the patients had had fresh citrus fruit for six months or more prior to admission.

Clinical Signs of Vitamin C Deficiency.—It was difficult to date the exact time of onset of the clinical disease. All of the patients complained of fatigue, weakness, and anorexia for months or years and had noted bruises usually related to slight trauma for a few weeks or several years before admission. Most of the patients said that their present illness began one or two months before admission with the onset of dull aching in the legs and swelling and pain in the knee or ankle joints. These symptoms were followed by the appearance of purplish discolorations about the affected joints, extending up and down the legs and arms and occasionally over the abdomen and face. Later, the subjects with natural teeth noted painful, swollen gums which interfered with mastication and from which blood oozed. With the onset of these major symptoms, the severity of the dyspnea, weakness, dizziness, lethargy, and anorexia increased, and many patients were aware that the color of their urine became dark red.

The most characteristic clinical sign was the minute perifollicular hemorrhage (see Plate I). It was found in sixteen subjects (Cases 2, 4 to 10, and 12 to 19), usually on the extensor surfaces of the legs or arms.

TABLE II. INITIAL EXAMINATION OF PERIPHERAL BLOOD ON ELEVEN PATIENTS WITH Scurvy Carried Out in Hematologic Laboratory of Cincinnati General Hospital

CASE	PATIENT	SEX	AGE	YEAR	R.B.C. (MIL- LIONS)	Hb. (GM.)	RETICU- LOCYTES (%)	PLATE- LETS		HEMATO- CRIT	M.C.V.	M.C.H.	M.C.H.C.	W.B.C.	DIFFERENTIAL W.B.C.				BASO- PHILES
															SEG- MENTED NEUTRO- PHILES	LYMPHO- CYTES	MONO- CYTES	EOSINO- PHILES	
9	J. M.	M	84	1943	2.68	9.5	6.6	205,920	29.5	110	35	32	32	10,500	64	27	8	1	0
10	J. K.	M	63	1943	3.03	10.6	5.2	178,500	31	102	35	34	34	3,300	82	11	6	1	0
11	W. B.	M	73	1944	1.74	5.8	1.0	205,320	17	98	33	34	34	4,450	79	17	2	2	0
12	L. F.	M	45	1942	2.18	6.3	5.0	120,000	21	97	29	30	30	12,600	76	14	10	0	0
13	A. S.	M	46	1944	2.25	5.7	3.5	157,000	18.5	82	25	31	31	2,700	81	8	10	0	0
14	C. K.	M	68	1944	2.81	9.2	8.4	252,900	30	107	33	31	31	5,400	69	20	8	2	1
15	J. H.	M	63	1944	3.30	9.7	1.0	303,600	30	91	29	32	32	9,750	80	14	6	0	0
16	C. G.	M	75	1944	1.88	7.8	10.1	475,000	21.5	115	41	36	36	3,750	64	33	3	0	0
17	H. E.	M	57	1945	2.73	8.5	5.0	—	26	95	31	33	33	5,000	60	30	7	3	0
18	C. M.	M	75	1945	2.46	7.5	5.7	270,000	26	105	30	29	29	5,200	72	15	10	2	1
19	T. K.	M	66	1945	2.46	7.0	8.2	408,000	23	94	28	30	30	5,500	52	39	8	1	0

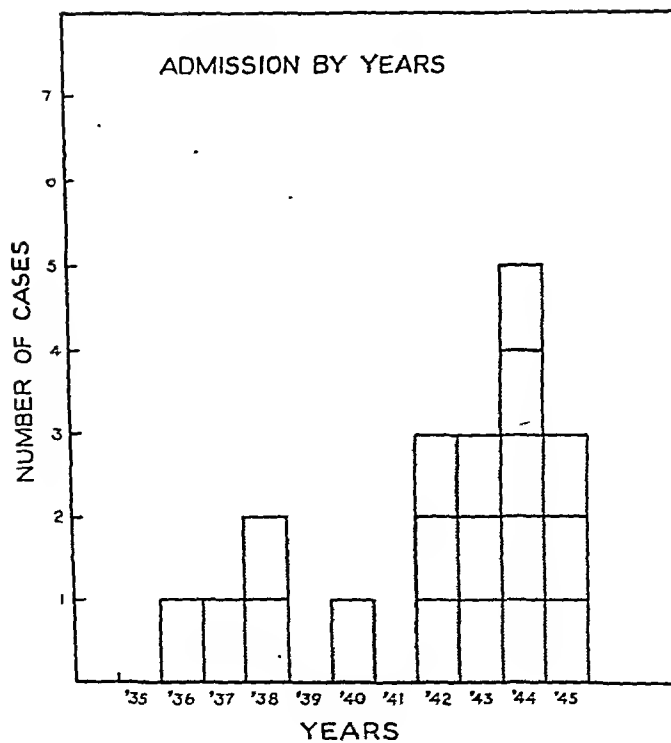
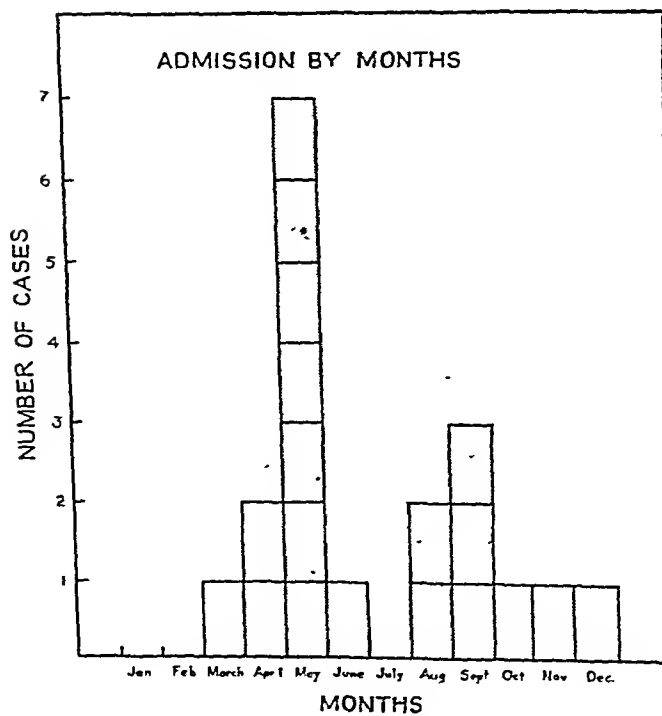


Fig. 1.



Seventeen patients (Cases 1, 3, 4, and 6 to 19) had purpura and ecchymoses ranging from a few millimeters to 20 cm. in diameter (Plate II, A and B). These ecchymoses were most common around the knees, ankles, and wrists and in the popliteal spaces. In many instances they occurred along superficial scratches and abrasions of the skin. Thromboses of saphenous, popliteal, femoral, or cephalic veins often were associated with the larger ecchymoses.

Four patients (Cases 11, 14, 15, and 19) had deep subcutaneous or intramuscular hemorrhages—hard, tender, deep masses, stretching the overlying skin of the arm or leg. Nine subjects (Cases 1, 4 to 8, 10, 11, and 15) had tender knee and ankle joints; five (Cases 1, 4, 6, 7, and 10) had moderately swollen joints; and in one (Case 5) the knee joint capsule was so tense with fluid that aspiration was done. Thirty cubic centimeters of grossly bloody fluid were removed.

Acute gum lesions were found in thirteen (Cases 2, 4 to 10, 12 to 15, and 19) of the fourteen patients (see Plate III, A and B) who had teeth or remnants thereof. No gum lesions occurred in those who were edentulous. The interdental papillae and gingival margins were greatly swollen, blue-red, tender, and highly friable. In some patients the swelling was so great that the teeth were nearly covered by gum tissue (Plate IV, A). The color changes were distinctive, even when the subject had a hemoglobin level of 6 or 7 Gm. The breath usually was foul, and the gums oozed blood following slight trauma; however in no patient was there spontaneous hemorrhage or blood loss in excess of a few cubic centimeters daily.

The patients generally had sallow, dirty grayish-yellow cadaveric complexions. Their hands and feet were cyanotic and cold, and in five subjects (Cases 5, 13, 14, 18, and 19) long thin splinter hemorrhages were observed under the nails (Plate IV, B). The sclerae were slightly icteric, and the ankles were edematous.

Twelve patients (Cases 2 to 4, 6, 7, 10 to 13, 15, 16, and 19) had temperature elevations ranging between 99.6 and 102° F., pulse rates ranging from 90 to 120 beats per minute, and respirations from 24 to 32 per minute. Blood pressures were normal or low in all except three subjects (Cases 1, 11, and 19) whose blood pressures were 195/100, 170/100, and 190/100, respectively, on admission and 155/80, 140/80, and 140/90 six days later just before they received anti-scorbutic medication.

Associated Vitamin Deficiency Diseases.—Since the diets of most of these individuals were deficient in more than one essential nutrient, evidence of vitamin deficiency diseases other than scurvy was common.

Seven patients (Cases 2 to 4, 10, 12, 15 and 19) had mild peripheral neuritis of thiamine deficiency; four (Cases 4, 10, 12 and 18) had fiery red glossitis of niacin deficiency; and two (Cases 3 and 16) had the magenta-colored glossitis and cheilosis of riboflavin deficiency. Five patients (Cases 2, 5, 8, 13, and 16) had follicular hyperkeratoses of the arms, legs, and buttocks, interpreted as evidence of vitamin A deficiency, and one (Case 8) had a healed rachitic chest deformity.

Plate I.

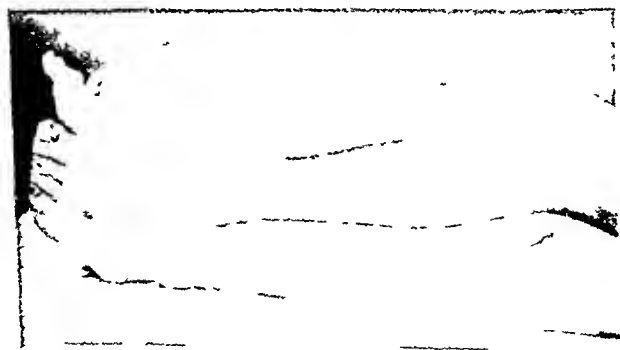
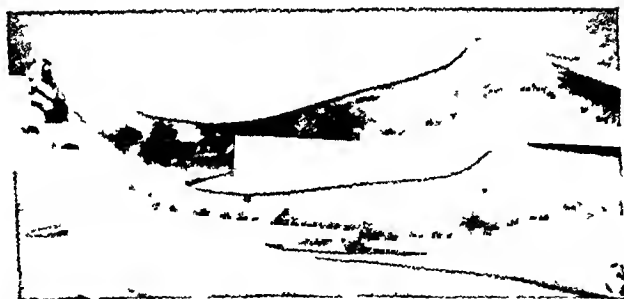


Plate II.

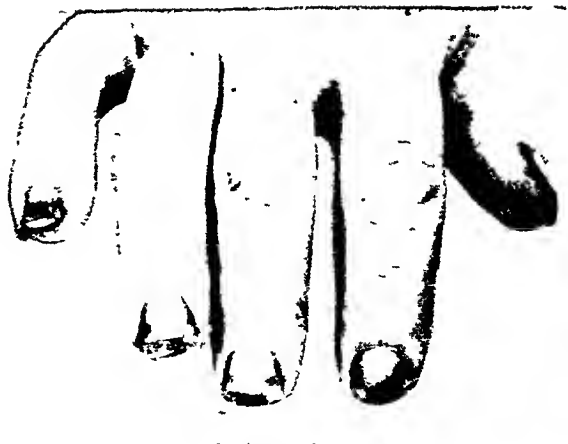
Plate I—Patient 8. Perifollicular hemorrhages

Plate II—Patient 16. A, Massive scorbutic eccymoses. B, Same area as A after sixteen days of therapy with vitamin C.

A.

Plate III.

B.



A.

Plate IV.

B.

Plate III.—Patient 9. A, Blue-red swollen gum about the remains of a tooth. B, Same gum area shown in A after twenty days of vitamin C therapy.

Plate IV.—Patient 2. A, Swollen spongy necrotic gums in a Negro with scurvy. B, Splinter hemorrhages in a patient with scurvy. These hemorrhages are similar to those observed in Patient 14.

TABLE III. BILE PIGMENT STUDIES, LIVER FUNCTION TESTS, AND SERUM IRON DETERMINATIONS

CASE	PATIENT	ICTERIC INDEX	QUALITATIVE VAN DEN BERGH REACTION	URINE UROBILINOGEN (DILUTION)	STOOL UROBILINOGEN (MG./24 HR.)	URINE BILIRUBIN (MG./24 HR.)	PROTHROMBIN TIME (SEC.)	SERUM PROTEIN (GM./100 G.C.)	SERUM ALBUMIN (GM./100 G.C.)	SERUM GLOBULIN (GM./100 G.C.)	CEPHALIN FLOCCULATION (IN 48 HR.)	BROM-SULFALIN (% IN 1/2 HR.)	SERUM IRON (MG./%)
1	J. B.	20	Indirect	+	0	0	22 (15)*	6.0	3.2	2.8			
4	G. F.	12	Indirect	1/16	0	0	18 (15)	5.18	2.43	2.75	+++	10	
9	J. M.	12			0	0	15 (15)	5.98					
10	J. K.	12	Biphasic† and indirect	1/102†	0	0	18.5 (14)	5.5	2.9	2.6			
11	W. B.	22	Biphasic† and indirect										
12	L. F.	10	Biphasic† and indirect										
13	A. S.	12	Indirect	1/32	0	0	15 (15)	4.3	2.6	1.7	++	<5	
14	C. K.	16	Biphasic† and indirect	1/64	0	0	20 (17)	6.08			+	<5	
15	J. H.	20	Biphasic† and indirect	1/32	0	0	16.5 (17)				+	<5	
16	G. G.	15	Indirect	1/128	0	0	17 (17)				+	<5	0.116
17	H. W.	10	Biphasic† and indirect	1/16	336	0	15 (15)	6.5			+	<5	0.083
18	C. N.	11	Biphasic† and indirect	1/64	397	0	16 (15)				+	15	0.072
19	T. K.	13	Biphasic† and indirect	1/32	945	0	14 (15)	6.37			+	15	

*Normal value for the day.

†Reaction delayed beyond five minutes.

TABLE IV. DIFFERENTIAL BONE MARROW C

PATIENT	POLY-MORPHO-NUCLEAR LEUCOCYTES (%)	METAMYELOCYTES (%)	MYELOCYTES C (%)	MYELOCYTES B (%)	MYELOCYTES A (%)	MYELOBLASTS (%)	LYMPHOCYTES (%)	M C (%)
J. M.	47.5	30.5	14.5	1.0			2.5	
J. K.	34.5	24.5	21.0	4.5	1.5	1.5	1.5	
W. B.	39.5	32.5	9.0	1.0	1.0	1.0	8.5	
L. F.	32.5	36.5	15.5	2.0	2.5		1.5	
A. S.	49.0	22.5	11.5	2.5			0.5	
C. K.	53.0	26.0	11.0	1.5			1.0	
J. H.	43.5	32.5	6.5	1.5			4.5	
C. G.	42.0	39.0	9.0	1.0		2.0	2.0	
H. E.	42.0	21.0	13.5	8.0	1.0	0.5	4.5	
C. N.	41.5	22.5	13.5	5.0	0.5	0.5	3.5	
T. K.	38.5	26.0	19.5	1.0	0.5		5.5	

TABLE V. BONE MARROW OF PATIENTS WITH S

PATIENT		POLY-MORPHO-NUCLEAR LEUCOCYTES (%)	METAMYELOCYTES (%)	MYELOCYTES C (%)	MYELOCYTES B (%)	MYELOCYTES A (%)	MYELOBLASTS (%)	LY C (%)
J. H.	Before vitamin C	43.5	32.5	6.5	1.5			
	Vitamin C 10 days	47.5	25.0	15.0	0.5			
T. K.	Before vitamin C	38.5	26.0	19.5	1.0	0.5		
	Vitamin C 7 days	34.5	23.5	17.0	1.5	0.5	0.5	
W. B.	Before vitamin C	39.5	32.5	9.0	1.0	1.0	1.0	
	Vitamin C 20 days	55.5	23.5	14.5	1.0			
C. G.	Before vitamin C	42.0	39.0	9.0	1.0		2.0	
	Vitamin C 4 days	37.5	34.5	15.0				
	Vitamin C 9 days	39.5	39.0	13.0	1.0			
	Vitamin C 25 days	53.0	28.5	7.5	2.5		0.5	

Analyses of the diets of thirteen subjects revealed that in three instances (Cases 4, 6, and 17) only vitamin C was grossly deficient; in five (Cases 10, 13 to 16) the vitamins of the B complex, vitamin C, and protein were grossly inadequate; and in the remaining five all essential nutrients were presumably eaten in inadequate amounts.

Laboratory Data.—No gross or occult blood was found in the urine, stool, or vomitus. Albumin was present in moderate amounts in the admission urine specimens of eight patients but cleared rapidly after bed rest was instituted. Icteric indices ranged from 10 to 22. The van den Bergh reactions were of the indirect or delayed biphasic type, and the urine contained an excess of urobilinogen but no bile. Stool urobilinogen content for twenty-four hours ranged from 336 to 945 mg. (normal, from 100 to 200 mg.). The prothrombin times were normal in seven of the eleven patients tested. Total serum proteins ranged from 4.31 to 6.68 Gm. per 100 c.c. The albumin values varied from 2.43 to 3.2 Gm. Cephalin flocculation tests were considered normal except in one

ON ELEVEN PATIENTS WITH ANEMIA OF SCURVY

EOSINO- PHILES (%)	EOSINO- PHILIC MYELO- CYTES (%)	BASO- PHILES (%)	PLASMA CELLS (%)	HEMO- CYTO- BLASTS (%)	MEGAL- OBLASTS PER 100 W.B.C.	EARLY ERYTH- RO- BLASTS PER 100 W.B.C.	LATE ERYTH- RO- BLASTS PER 100 W.B.C.	NORMO- BLASTS PER 100 W.B.C.	NUM- BER OF NU- CLEATED R.B.C. PER 100 W.B.C.
2.0	1.0		0.5		1.0	2.5	22.0	152.5	178.0
2.0	0.5		6.0		0.5	1.5	5.5	59.5	67.0
1.5			4.0	1.5	1.5	7.5	30.0	41.5	80.5
2.5			6.0		0.5	2.5	6.5	68.5	78.0
3.0	3.0	0.5	5.5	2.0		0.5	2.5	85.5	88.5
3.5			3.5	0.5		1.0	9.5	136.5	147.0
1.5	1.0	0.5	8.5		1.0	2.5	11.0	69.5	84.0
2.0		1.0					8.0	76.5	84.5
2.5	1.5	1.5	3.5		1.5	10.0	31.5	139.5	182.5
5.0	1.0	0.5	6.0			2.5	5.5	63.5	71.5
5.0	0.5		3.5		1.5	4.5	22.5	75.0	103.5

BEFORE AND AFTER ADMINISTRATION OF VITAMIN C

MONO- CYTES (%)	EOSINO- PHILES (%)	EOSINO- PHILIC MYELO- CYTES (%)	BASO- PHILES (%)	PLASMA CELLS (%)	HEMO- CYTO- BLASTS (%)	MEGALO- BLASTS PER 100 W.B.C.	EARLY ERYTH- ROBLASTS PER 100 W.B.C.	LATE ERYTH- ROBLASTS PER 100 W.B.C.	NORMO- BLAST PER 100 W.B.C.	NUMBER OF NU- CLEATED R.B.C. PER 100 W.B.C.
	1.5	1.0	0.5	8.5		1.0	2.5	11.0	69.5	84.0
0.5	1.5	1.5	0.5	5.0		0.5	4.5	32.5	223.0	261.5
	5.0	0.5		3.5		1.5	4.5	22.5	75.0	103.0
	0.5	1.5	0.5	2.0	0.5	1.5	8.0	18.5	167.0	195.0
0.5	1.5			4.0	1.5	2.5	15.0	60.0	83.5	161.0
0.5		1.5	0.5	0.5	0.5		1.0	2.5	59.0	62.5
	2.0		1.0	2.0				8.0	76.5	84.5
	1.0	0.5	1.0	6.5			1.0	8.5	56.5	66.0
	0.5	2.5	0.5	2.5			1.0	7.0	52.0	60.0
	1.5	2.5	1.0	2.5				1.0	25.0	26.0

patient (Case 11); bromsulfalein retention at thirty minutes varied from 5 to 15 per cent (Table III). Gastric analyses showed hypochlorhydria or achlorhydria after histamine stimulation. The plasma vitamin C content was 0.0 mg. per cent. Values for serum iron were normal; they ranged from .072 to 0.116 mg. per cent.

Hematologic Data.—The peripheral blood characteristics for all patients are summarized in Tables I and II. Two patients with mild scurvy of recent onset (Cases 5 and 6) had erythrocyte counts over 3.5 million and hemoglobin values over 12 Gm. The erythrocyte counts in the seventeen more severely ill subjects ranged from 1.74 to 3.44 million and the hemoglobin levels, from 5.8 to 10.5 Gm. The cells were normochromic and normocytic or moderately macrocytic; they varied only slightly in size and shape. The admission white cell counts were under 6,000 in twelve of the nineteen patients. Some were as low as 2,500. Differential white cell counts were normal. Platelets occurred in normal or moderate reduced numbers. Initial reticulocyte counts ranged from

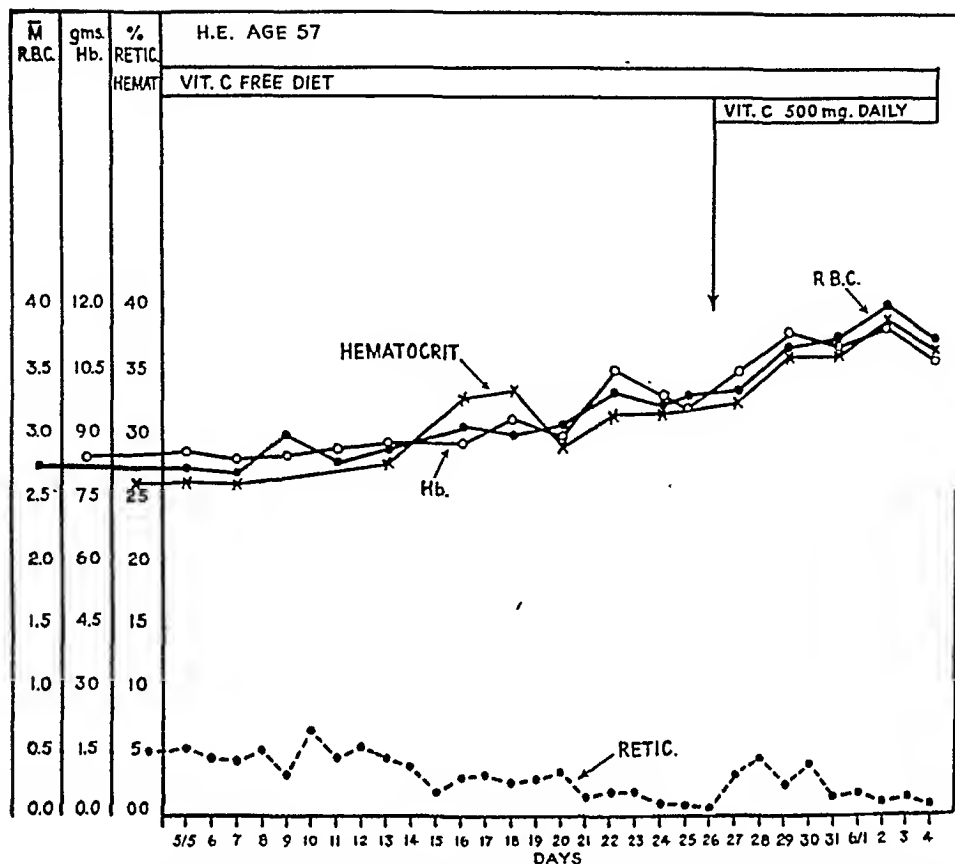


Fig. 3.—Illustrating the gradual hematologic recovery of a patient with moderately severe scurvy of short duration, following rest in bed. Administration of vitamin C appears to have been associated with a small increase in reticulocytes and an acceleration of erythrocyte and hemoglobin regeneration.

H. E., (Case 17), a 57-year-old white man, was admitted to the Cincinnati General Hospital May 5, 1945. For one week prior to admission he had had pain in bones and joints, swelling of the left elbow and left knee, and painful swelling of the left thigh with purplish discoloration of the skin overlying this area. Similar swelling and pain in the left upper extremity had occurred several days prior to admission. Small red-blue spots also had appeared on the arms and legs, and he had become weak and dizzy.

He had had Jamaica ginger paralysis in 1930. Since this time he had been living alone, moving from bed to chair, and cooking for himself. His diet consisted of two meals a day. For breakfast he had two eggs, a bowl of cereal, and four pieces of bread. Lunch consisted of one quart of milk, graham crackers, and whole wheat bread. He never ate citrus fruits and very seldom ate green vegetables. He attempted to make up for this lack of vitamins by taking Hemo.

The temperature was 98° F.; pulse, 100; blood pressure, 140/90. There were extensive ecchymoses over the left popliteal and left antecubital areas, extending up and down the left leg and left arm. Phlebothrombosis was present in both areas, and considerable collateral venous circulation was observed over the left shoulder. There was fluid in the left knee. Small areas of purpura and perifollicular hemorrhages were noted over the arms and legs. There were splinter hemorrhages under the nails. Teeth were absent, and gums were essentially normal. Neurologic examination showed a spastic quadriplegia with muscle atrophy

(Continued on opposite page.)

3 to 10 per cent except in two patients (Cases 11 and 15) in whom initial counts of 1 per cent were obtained.

The bone marrow appeared to be moderately hypercellular in five patients (Cases 9, 10, 11, 13, and 19), normally cellular in five (Cases 12, 14, 15, 17, and 18), and moderately hypocellular in one subject (Case 16). Differential counts of nucleated cells showed a relative increase in erythrocyte progenitors with the majority of these cells at the normoblast and late erythroblast stages of development (Table IV). In one patient (Case 11) who had the most severe anemia we have encountered, 11 per cent of the young red cells were megaloblasts and early erythroblasts. In all marrow specimens, the granulocyte series was cytologically normal or showed a slight shift to young cell forms.

CLINICAL COURSE AND RESPONSE TO THERAPY

The eleven patients under our direct observation were kept on the vitamin C restricted diet for the period of hospitalization and were given placebos until their safety seemed jeopardized or until improvement occurred from bed rest alone. The shortest period of preliminary observation was two days; the longest, twenty-one days.

Two patients (Cases 17 and 18) showed slow but definite clinical and hematologic improvement on bed rest alone. On admission they were classed as mildly or moderately ill. Even though their plasma vitamin C remained 0.0 mg. per cent, their perifollicular hemorrhages and ecchymoses cleared over a period of from fourteen to twenty-one days, and they felt subjectively improved. Icteric indices and urine urobilinogen concentrations reached normal values in the same period of time. In one subject (Case 18) the erythrocytes and hemoglobin had increased 500,000 cells and 2.4 Gm., respectively, at the end of twelve days, and reticulocytes had fallen from 5.7 to 1.6 per cent. Administration of vitamin C at this time did not seem to accelerate blood formation. One subject (Case 17) showed much slower blood regeneration. During a period of twenty-one days, the erythrocyte count increased from 2.75 to 3.27 million and the hemoglobin from 8.5 to 9.6 Gm. Reticulocytes fell from 5.0 to 0.8 per cent. Bone marrow aspiration repeated at this time showed

of the extremities. The calves were tender, and there was diminished perception of light touch and vibration over the legs and feet. No jaundice was noted and the skin color was normal.

For data concerning blood examinations, liver function tests and bile pigment studies, and bone marrow examinations, see Tables II, III, and IV. Bleeding time was two and one-half minutes; clotting time, six minutes. Plasma ascorbic acid was 0.0 mg. per cent; blood urea nitrogen, 14 mg. per cent.

The patient was placed on the vitamin C restricted diet and was observed for twenty-one days. The lesions of scurvy slowly cleared, and the erythrocyte count and hemoglobin level gradually rose from 2.73 to 3.23 million and from 8.5 to 9.8 Gm., respectively. Plasma vitamin C level was still 0.0 mg. per cent.

Administration of vitamin C, 100 mg. five times a day by mouth, was begun May 26, 1945. A very slight elevation of reticulocytes occurred and possibly some increase in the rate of erythrocyte regeneration.

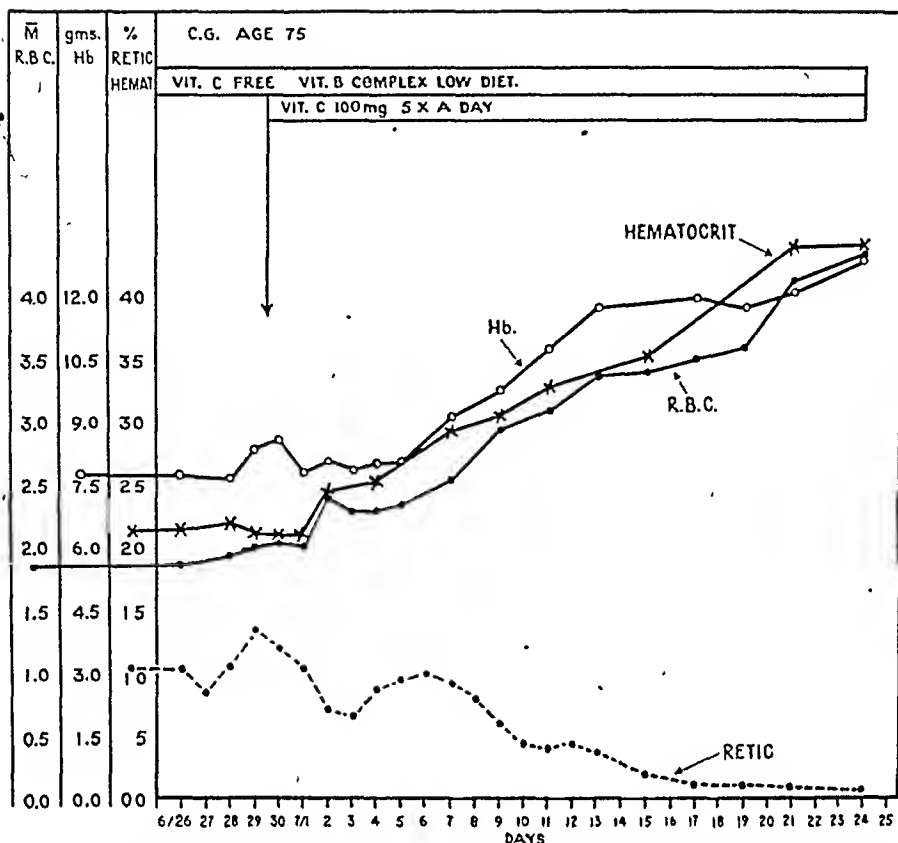


Fig. 4.—A patient with severe scurvy. The erythrocyte count and hemoglobin were unchanged before vitamin C therapy, but the patient's physical condition deteriorated so rapidly that vitamin C had to be administered as a life-saving measure. Thereafter, the erythrocytes and hemoglobin regenerated rapidly as the reticulocytes decreased.

C. G. (Case 16), a 75-year-old white man, was admitted to the Cincinnati General Hospital June 26, 1944, because of weakness, aching legs, and purplish discoloration of the skin for three months. His wife had died one year before admission, and he had had no citrus fruit or tomatoes since that time. He prepared his own meals. Breakfast consisted of two eggs, bacon, and coffee. For lunch he ate soup, beans, and crackers. He had a sandwich and sometimes nothing for supper.

The temperature was 101° F.; pulso, 80; respirations, 24; blood pressure, 110/50. He was a lethargic old man, and the skin was a dirty lead gray color. There were perifollicular hemorrhages on the arms. The entire right leg to the crest of the ilium was covered by confluent ecchymoses. The left leg was less severely involved. The sclerae were icteric. He was edentulous and the tongue was smooth, fissured, and pale. The only abnormality on neurologic examination was absence of the knee and ankle jerks bilaterally.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. No vitamin C was found in the plasma.

The patient was placed on the control diet. By the fourth hospital day the blood pressure had fallen to 95/50 and he had become very lethargic and confused. Administration of 500 mg. of vitamin C intramuscularly in divided doses was begun. One day thereafter the temperature was normal, blood pressure, 115/55, and the patient was much less confused. Rapid improvement continued even though he developed cheilosis and a magenta tongue which responded satisfactorily to riboflavin. The icteric index reached normal in twelve days, and the erythrocyte count and hemoglobin reached 4.3 million and 12.9 Gm., respectively, in twenty-six days. Blood pressure at discharge was 130/70.

depressed activity of all marrow elements. After vitamin C was administered, a small rise in reticulocytes occurred, and erythrocyte and hemoglobin regeneration appeared to be accelerated (see Fig. 3). Complete recovery did not occur until the subject was given an adequate diet.

Nine patients (Cases 9 to 16 and 19) classed on admission as severely ill did not show any clinical improvement during the observation period. The ecchymoses and perifollicular hemorrhages changed from dark reddish-purple to dark brownish-purple but did not fade or spread. Gingival lesions remained unchanged or progressed. Blood pressures, frequently at a low level on admission, fell. Icteric indices and urine urobilinogen concentrations rose. Two of the patients developed Cheyne-Stokes type of respiration. Mental torpor and cyanosis deepened, and the cadaveric gray skin color became more prominent. Six of these subjects remained unchanged hematologically (Cases 9 to 13 and 16) during the control period (see Figs. 4 and 5). Three patients (Cases 14, 15, and 19) had a rapid decline in erythrocytes and hemoglobin and an increase in reticulocytes as their clinical condition grew worse (see Figs. 6 and 7). When signs of impending shock became alarming, vitamin C was given in doses of 100 mg. five times a day, orally or intramuscularly, depending on the clinical condition of the patient.

All of these nine patients improved within from twenty-four to forty-eight hours after administration of vitamin C. Nail beds became pink, the cadaveric gray color of the skin disappeared, the blood pressure rose, and the Cheyne-Stokes type of respiration was replaced by normal breathing. Those patients who had temperature and pulse elevations before the administration of vitamin C had normal temperatures and pulse rates within from one to three days afterward.

Perifollicular hemorrhages disappeared after from two to three days leaving the affected hair follicles hyperkeratotic and pigmented. The engorged, blue-red swollen gums began to clear after forty-eight hours. As the swelling receded, the gums retracted from the teeth and appeared pale, scarred, pitted, and thickened. Infected pockets between teeth and gum never healed completely. Joint swelling and pain disappeared over a period of from three to five days, and major ecchymoses disappeared over a period of from two to three weeks leaving large areas of pigmentation in their wake. Icteric indices and urine urobilinogen concentrations fell to normal levels in four days (Cases 14 and 15), six days (Case 19), and twelve days (Cases 11 and 16). Four days after vitamin C therapy the stool urobilinogen content had decreased from 945 to 265 mg. per 24 hours in the one subject (Case 19) in whom the test was repeated.

In two of these nine subjects (Cases 11 and 12) reticulocytes rose from 2 to 19 per cent and from 5 to 10 per cent by the tenth and sixth days, respectively (see Fig. 8). In seven patients the high reticulocyte levels were maintained or rose slightly between the fourth and seventh days, subsequently falling slowly to normal.

Red cell and hemoglobin increases began from four to seven days after vitamin C administration, and after three weeks the levels had reached 3.5

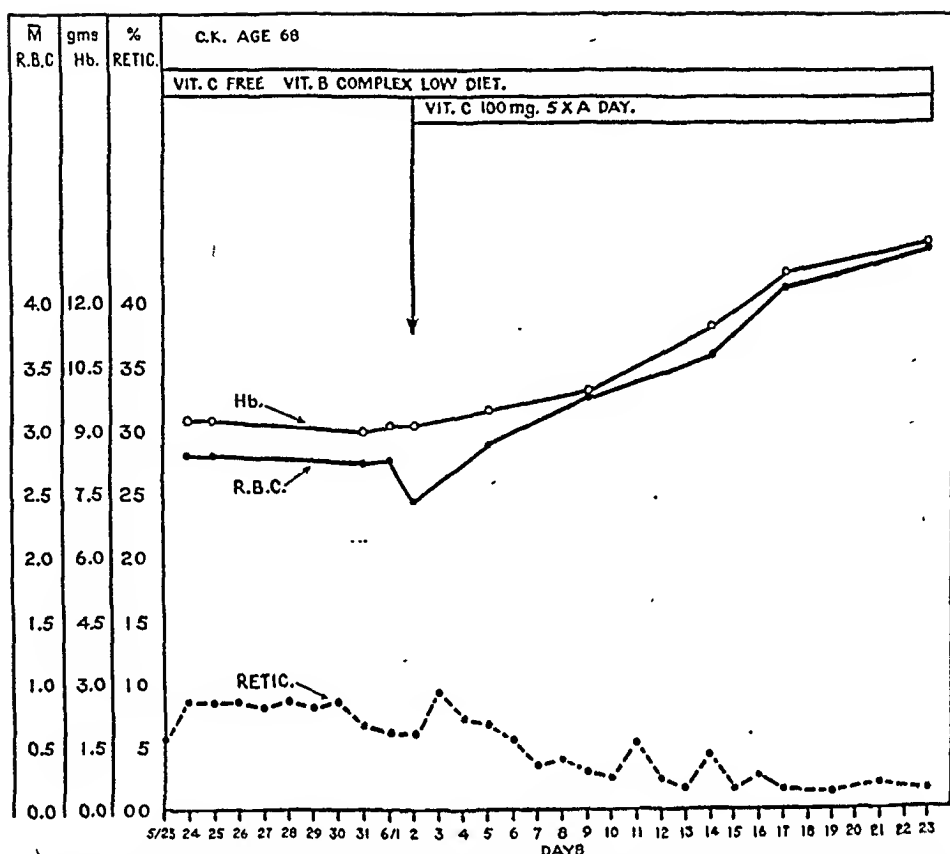


Fig. 5.—The course of a patient with severe scurvy on the vitamin C free diet. The erythrocytes and hemoglobin values were stationary but his clinical condition grew worse during the eleven days prior to the administration of vitamin C. Thereafter, he made a rapid clinical and hematologic recovery.

C. K. (Case 14), a 68-year-old single white man, was admitted May 22, 1944, because of intermittent leg and joint pain and sore gums of several months' duration. Six weeks before admission the right knee swelled and the popliteal area became ecchymotic. Pin-point purplish spots over the arms came and went during this period. The gums had been sore intermittently, and the teeth had been falling out for seven years. In 1943 he had been on the vascular service for phlebothrombosis and had been treated with vitamin C because of purpura over both lower extremities. His diet consisted of sausage meat, bread, coffee, and potatoes. He said he had never eaten fruit or green vegetables.

The temperature was 99° F.; pulse, 70; respirations, 24; and blood pressure, 94/55. The mouth contained one loose tooth snag surrounded by elevated, soft blue-red hemorrhagic mucous membrane. Otherwise the oral mucous membrane was normal. The tongue was smooth, pale, and clean. There were many small punctate hemorrhages on the dorsal surfaces of the arm and legs. There was a massive ecchymosis associated with phlebothrombosis behind the right knee. The skin was a sallow lead gray color. There were long, old splinter hemorrhages under the fingernails. The sclerae were icteric. The right leg was edematous.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. Gastric analysis showed 32° free acid after histamine stimulation. Bleeding time was one and one-half minutes; coagulation time, three and one-half minutes. Tourniquet test was negative after five minutes. No vitamin C was found in the blood plasma. Electrocardiogram showed low voltage upright T waves in Leads II and III; T₁ was diphasic; T₄, inverted.

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million and 12.5 Gm. or more in all patients. Repeated bone marrow aspirations on four subjects (Cases 11, 15, 16, and 19) after vitamin C was administered for four or more days showed reversion to normal marrow. In two instances a striking increase in the per cent of normoblasts and late erythroblasts (Table V) was found one week after the administration of vitamin C.

Of the eight persons whose hospital records were reviewed, one (Case 8) whose illness was mild showed complete healing of gum lesions, disappearance of perifollicular hemorrhages, and rapid rise in red blood cell count and hemoglobin on bed rest without benefit of crystalline vitamin C or citrus fruits. The other patients received vitamin C in doses of from 300 to 1,000 mg. per day after varying periods of study, during which vitamin C was restricted in the diet. Before vitamin C was administered, the blood pressure of one patient (Case 4) had fallen to 60/20. He developed signs of thrombosis of a branch of the right middle cerebral artery. Except for the residual hemiparesis, he made an uneventful recovery. Another subject (Case 1) received vitamin C only after he had experienced an episode of severe dyspnea and cyanosis associated with a precipitous fall in blood pressure. In spite of heroic efforts, he died six hours afterward in a similar episode. Autopsy was consistent with scurvy and revealed no other cause of death. The rest of the patients made complete and uneventful recoveries in the manner previously described.

DISCUSSION

The clinical observations recorded in this group of patients are similar to those previously reported,⁹⁻¹¹ but several features seem worthy of special emphasis. The general appearance of patients with severe scurvy is distinctive. Stasis cyanosis in the extremities, sallow dirty gray cadaveric skin color, somnolence, lethargy, and hypotension appear insidiously and are the prodromas of peripheral vascular collapse which may occur suddenly without further warning. Cheyne-Stokes type of respiration occurs particularly in patients with arteriosclerotic cerebrovascular disease and anemia. All of these vasomotor abnormalities disappear within from twenty-four to thirty-six hours after the oral or parenteral administration of adequate amounts of vitamin C. Although the exact mechanism responsible for these changes is unknown, it should be noted that a direct relationship has been reported to exist in guinea pigs and

After a week on the vitamin C restricted diet, the patient developed respiratory irregularity and peripheral cyanosis. On the tenth day he became very dyspneic even after the slight exertion of sitting up in bed. When resting, the respirations were of the Cheyne-Stokes type. He was confused and somnolent. The blood pressure was 80/50. Administration of vitamin C, 500 mg. daily in divided doses intramuscularly, was begun. Thirty-six hours thereafter the respirations were regular except for an occasional apneic period. The blue-red color about the tooth snag had faded, and the swelling had decreased. The lead gray color of the skin and cyanosis of the nails had disappeared. Blood pressure was 108/60. Three days after vitamin C, respirations were entirely normal, and the patient was well orientated. Blood pressure was 115/65. In four days the icteric index was 5, and urine urobilinogen was within normal limits. The gum and skin lesions healed completely in twelve days. Blood counts were normal in three weeks.

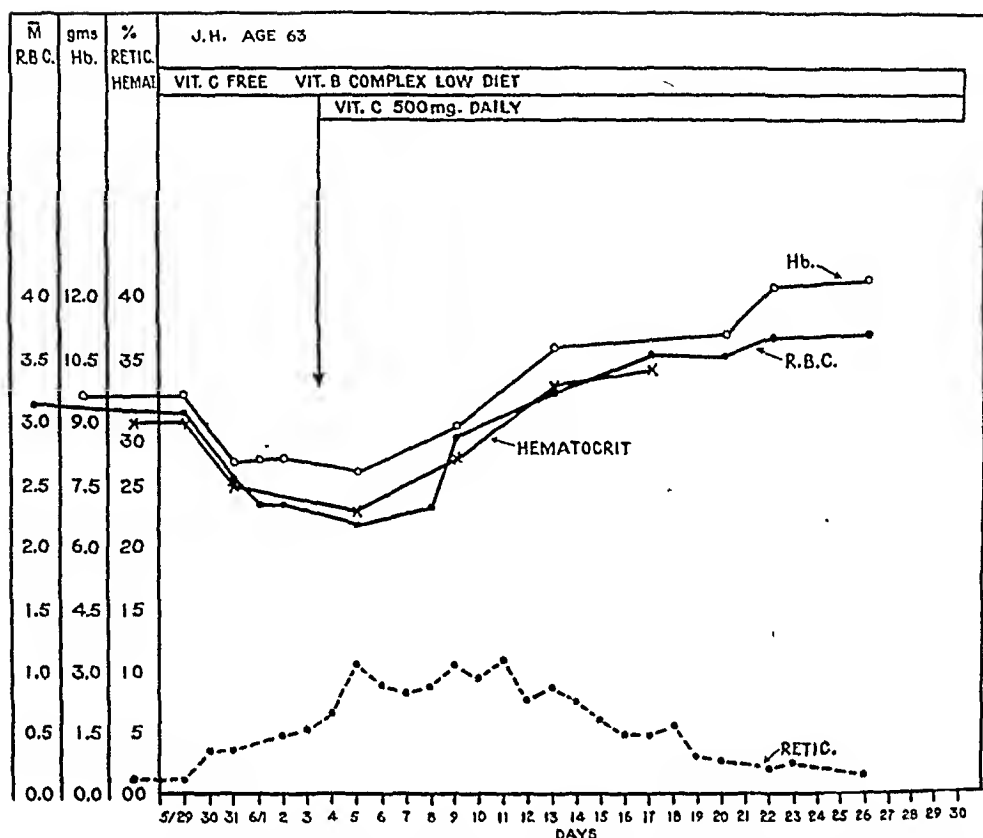


Fig. 6.—A patient with severe scurvy whose erythrocytes and hemoglobin declined and reticulocytes rose as his clinical course grew worse prior to vitamin C therapy. Thereafter, he made a rapid hematologic and clinical recovery.

J. H. (Case 15), a 63-year-old white man, was admitted to the Cincinnati General Hospital May 27, 1944, because of swelling of the legs and pain in the knees of six months' duration. For one year he had noticed easy bruising and for the previous six months numbness and tingling in the feet and legs. He ate oranges or grapefruit only when the cost was 15 cents a dozen. Breakfast consisted of coffee, canned milk, and doughnuts; dinner, soup, occasionally meat, potatoes, beans, bread, and beer; supper, a sandwich and beer. His wife had died in 1918, and he had been cooking for himself ever since.

The temperature was 101° F.; pulse, 116; respirations, 22; and blood pressure, 108/74. There was an 8 by 6 by 4 cm. deep-seated hematoma on the right forearm, and there were large ecchymotic areas as well as perifollicular hemorrhages over the thighs and lower legs. The ankles were moderately edematous. The sclerae were icteric and the mucous membranes pale and slightly cyanotic. The gums were swollen, blue-red, and spongy and bled easily. Neurologic examination showed only absent Achilles jerks and diffuse tenderness over the lower legs, especially in the regions of the ecchymoses. Later, distinct stocking type of hyperesthesia was found.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. No vitamin C was found in the plasma. X-ray of the chest showed moderate congestion at the lung bases. The tourniquet test was positive.

The patient was fed the vitamin C restricted diet during the entire hospital stay. After eight days the blood pressure had fallen to 90/50, and he was becoming confused. The skin was a sallow gray color. The erythrocyte count and hemoglobin level had fallen, and the reticulocyte count had risen to 5 per cent. Vitamin C was begun in dosage of 500 mg. a day orally, and the patient recovered over a period of three weeks. Four days after vitamin C was begun the blood pressure was 110/64. Icteric index was normal.

rats between the vitamin C stored in the adrenals and the synthesis of adrenocortical steroids.^{12, 13}

In 1930 Mettier, Minot, and Townsend⁹ concluded that scorbutic anemia responded specifically when orange juice was administered but did not respond to iron or purified liver extract. They showed that the erythrocytes were normocytic normochromic or moderately macrocytic hyperchromic, in contrast to previous teaching that the cells generally were hypochromic. They found the bone marrow moderately hyperplastic and normoblastic and noted an increase in cellularity due principally to an increase in normoblasts after vitamin C was administered.

Since 1930 numerous observers¹⁴⁻²² have reported hematologic studies on one, two, or three persons with scurvy which had occurred naturally or had been induced experimentally.²³ The most comprehensive recent study involved fifty-three scorbutic patients in Edinburgh, Scotland.²⁴ Much of this work seems to indicate that scurvy and anemia do not necessarily coexist, that experimental vitamin C lack does not interfere with blood formation, and that patients with naturally occurring scurvy and anemia show erythrocyte and hemoglobin regeneration while on a vitamin C free but otherwise adequate diet. The bone marrow has been described as hyperplastic with normoblastic maturation arrest,⁹ as hypoplastic,^{21, 25} and as megaloblastic.¹⁶ In short, the inference is that vitamin C is not essential for normal hematopoiesis and that hemorrhage, lack of iron, and some unknown vitamin B complex or other deficiency state account for scorbutic anemia.

The hematologic data gathered from our severely scorbutic patients while they were on a diet very low in vitamin C and low in the vitamins of the B complex corroborate many of the original observations of Mettier, Minot, and Townsend.⁹ Nine of our critically deficient patients either did not improve clinically or hematologically or became more anemic and debilitated on this diet. Striking hematologic and clinical recovery occurred after vitamin C alone was added to the experimental regime, much the same effect previously reported for orange juice.

In addition, almost all of our patients had signs suggesting accelerated blood destruction, namely, reticuloeytosis, slight or moderate jaundice, elevated urobilinogen output in urine and stool but no bile in the urine. All of these signs disappeared within from four to twelve days after vitamin C was administered. None of the patients had clinical evidence of hepatic dysfunction, and of twenty-nine liver function tests performed on eleven patients, only nine tests were definitely abnormal. Such hepatic dysfunction as was found by laboratory procedures was no more than might have occurred from chronic nutritional deficiency. Liver disease could scarcely have been responsible for all of the signs associated with the jaundice. These data suggest that hemolysis was partially responsible for the anemia and that this factor was rapidly eliminated by the administration of vitamin C. Since neither the degree of the anemia nor the degree of the jaundice could be correlated with the extent of visible ecchymoses, it seems possible that some of the hemolysis occurred in-

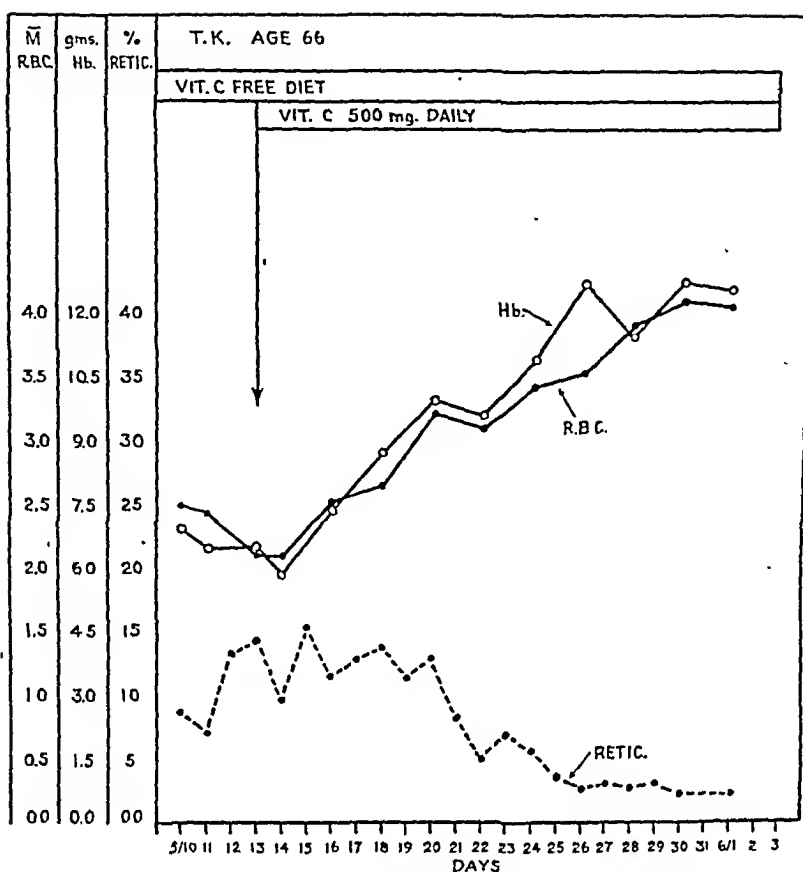


Fig. 7.—A patient with severe scurvy whose erythrocytes and hemoglobin declined and reticulocytes rose as his clinical condition grew worse prior to the administration of vitamin C. Thereafter, he made a rapid clinical and hematologic recovery.

T. K. (Case 19), a 66-year-old white man, was admitted to the Cincinnati General Hospital May 10, 1945, because of inability to walk, tenderness in the calves, and numbness in the feet. The appetite had been poor, and he had been eating nothing but vegetables, soup, cheese, bread, and beer for six months. Prior to that the chief articles of diet were beans, sauerkraut, and potatoes. Two years before admission he quit work because of weakness in the legs. He was living with his wife, but his daughter, who had been caring for the old folks, had left for the WACs one year before. He was unable to remember when he had last eaten citrus fruits or tomatoes.

The temperature was 100.6° F.; pulse, 90; respirations, 24; blood pressure, 190/100. The respirations were of Cheyne-Stokes type. The skin over the legs and thighs was covered with large, deep, dark purpuric spots with white firm centers. The gums were blue-red, swollen, and very tender. The sclerae were icteric. The skin was a dirty yellow-gray color, nail beds were cyanotic, and there were splinter hemorrhages under the nails. The chest was emphysematous. The patient was confused and disoriented. The arms and legs were somewhat spastic. All deep reflexes were hyperactive. There were bilateral positive Hoffmann reflexes and bilateral ankle clonus.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. The plasma vitamin C level was 0.0 mg. per cent; blood urea nitrogen 18 mg. per cent. Electrocardiogram showed myocardial dam-

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travascularly rather than solely in the ecchymoses, although this has not been proved.

The bone marrow was never as hyperplastic as one would expect in patients with pure hemolytic jaundice or the anemia of acute blood loss. In fact, the marrow sample obtained by sternal aspiration was definitely hypocellular in one patient who had distinctly elevated reticulocytes. If this specimen represented adequate sampling of all the marrow, then definite hypocellularity was present. White blood cell counts frequently were low. These observations suggest that there were significant etiologic factors other than hemolysis. All of these abnormalities disappeared when vitamin C alone was administered to patients whose previous course had been stationary or downhill. The conclusion seems inescapable that vitamin C reversed these abnormal processes and is essential for normal formation and maintenance of erythrocytes.

On the other hand, we too have found that patients with mild or moderate scurvy may have no anemia or may improve clinically and hematologically when they are put to bed and fed a vitamin C restricted diet. In fact, a normal blood picture has been found in twelve ambulatory patients with scurvy who have visited the Nutrition Clinic, Hillman Hospital, Birmingham, Ala.* Further observations from this clinic indicate that many nutritionally deficient persons may have repeatedly negative tests for vitamin C in the plasma for five years and yet develop neither clinical scurvy nor anemia.

The factors which cause the development of anemia in some persons with scurvy but not in others are not clearly understood. Certain considerations, however, help explain this variation in hematologic response. In the normal course of events deficiency diseases seldom occur as single entities. Deficient diets are seldom lacking in a single essential factor. The altered physiology induced by the deficiency of one essential nutrient may increase the need of the cells for the other essential nutrients and may exaggerate the pathologic response of the cells if a second or third deficiency occurs. As an example,

age, S-T₁ and S-T₂ depressed; QRS, .10 second; P-R interval, .20 second. Cerebrospinal fluid was normal.

The patient was placed on the vitamin C restricted diet for the entire hospital stay. After four days, periodic breathing was more pronounced and he was much more confused, the nail beds more cyanotic, and the blood pressure 140/90. Erythrocyte and hemoglobin levels had fallen and reticulocytes had increased to 14 per cent. He told the examining physician that he would not live until the next morning. Vitamin C in doses of 100 mg. five times a day was given by mouth May 14, 1945. After twenty-four hours the peripheral cyanosis and muddy color of the skin had disappeared. The gums were less swollen and were paler. Sclerae appeared to be less icteric. The patient was less confused and was breathing normally.

In six days the gums had completely healed and the purpuric areas had become brown. All signs of jaundice had disappeared and the icteric index was 5. The patient made a rapid recovery from scurvy, but neurologic manifestations were unaltered.

*These studies were supported by grants from Chas. Pfizer & Co., Inc., New York, N. Y.

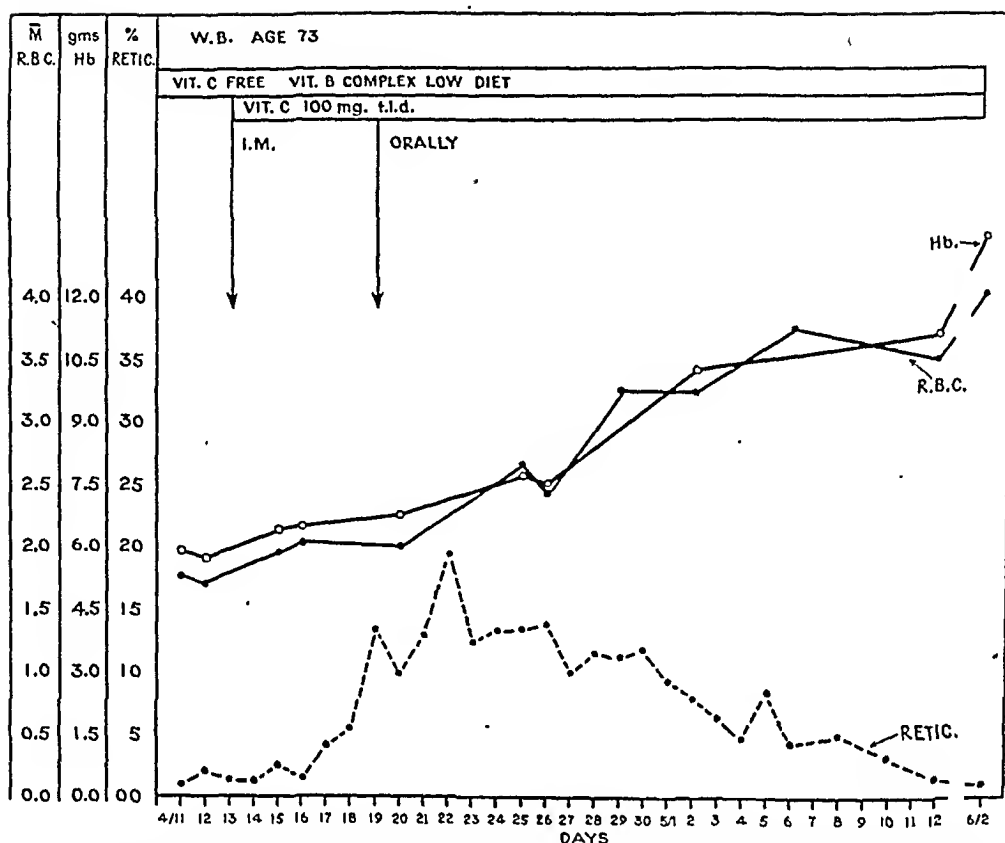


Fig. 8.—A patient with severe scurvy. Ten days after vitamin C was begun, reticulocytes had increased to a maximum of 19.2 per cent. Thereafter, the erythrocytes and hemoglobin increased rapidly. Such a reticulocyte response following the administration of vitamin C was observed in only one other patient.

W. B. (Case 11), a 73-year-old white man, entered the Cincinnati General Hospital April 4, 1944, because of pain and weakness in the knees. One year before he had noted black and blue marks about each knee which appeared without trauma. Recently these areas had become larger and the knee joints painful. He had never married and had always eaten in restaurants. He stated that he liked fruits and vegetables but could not afford to eat them.

His temperature was 99.8° F.; pulse, 98; respirations, 20; and blood pressure, 190/94. The inner aspects of the thighs and both knees were covered with confluent ecchymoses associated with phlebothrombosis in the popliteal regions. There were a few perifollicular hemorrhages over the tibiae and small subcutaneous ecchymoses on the right elbow, left forearm, right temple, and right upper eyelid. The mucous membranes were pale and the sclerae were jaundiced. Gums were normal, even around one tooth remnant. The liver was moderately enlarged but nontender. Signs of old left hemiplegia were found.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. The vitamin C level in the plasma was 0.0 mg. per cent. Blood urea nitrogen was 26 mg. per cent. Gastric analysis showed 22° of free hydrochloric acid after histamine stimulation. A barium enema and gastrointestinal series showed only distended bowel.

The patient received the vitamin C restricted diet during the entire period of hospitalization, and administration of vitamin C, 100 mg. three times a day intramuscularly, was begun April 13, 1944. Ten days later the reticulocytes had mounted to 19.2 per cent, and twelve days after administration of vitamin C the icteric index and urine urobilinogen values were normal. The patient was discharged June 2, 1944, in good condition.

a patient with severe vitamin C depletion may have no anemia until additional strain is placed on the bone marrow by a deficiency of extrinsic factor, protein, iron, or other unknown factors which may be necessary for normal hematopoiesis. Yet, after anemia has developed, the deficiency of the latter factors may not be serious enough to prevent a remission when large amounts of vitamin C are administered. In many deficient persons, bed rest which reduces metabolic requirements for all essential nutrients may be sufficient therapy to produce a clinical and hematologic remission. Depending on the interplay of multiple factors, morphologic differences in blood and bone marrow and varied therapeutic responses may readily occur in patients with scurvy and other deficiency states. For these reasons, our observations on patients who were critically ill with scurvy, anemia, and other deficiency diseases of long standing cannot be compared satisfactorily with data on human subjects in whom a single deficiency state, scurvy, has been produced experimentally²³ without the occurrence of anemia. For all these reasons, the stage is set for the accumulation of data which appear to be contradictory. The difficulties of establishing the exact relationship of vitamin C to the anemia of scurvy are multiplied. Our data indicate that a deficiency of vitamin C is the major factor in the development of scorbutic anemia such as was found in these patients, although the *modus operandi* remain unsettled.

SUMMARY AND CONCLUSIONS

1. The prodromal period and the clinical features of severe scurvy are described in nineteen scorbutic patients admitted to the Cincinnati General Hospital over the past ten years. Perifollicular hemorrhages, ecchymoses, swollen painful joints, and swollen blue-red tender gums occurred frequently. Blood pressures tended to be low, and many of the patients were bordering on vascular collapse.

2. All but two of the nineteen patients had anemia.

3. Complete hematologic studies were carried out on eleven patients. The anemia was normochromic and normocytic or slightly macrocytic. Persistent reticulocytosis, moderate leucopenia, and thrombopenia occurred frequently. Moderate icterus of the hemolytic type was noted in most of the patients.

4. The bone marrow appeared moderately hypocellular in one patient and normally cellular or hypercellular in the others. Differential counts of nucleated marrow cells indicated a relative increase in normoblasts and, to a lesser extent, in the younger members of the red cell series.

5. Two patients with mild or moderately severe scurvy improved clinically and hematologically after bed rest.

6. In nine severely scorbutic patients, despite persistent reticulocytosis, erythrocyte counts and hemoglobin levels were unchanged or fell while the patients were in bed on a vitamin C restricted diet.

7. Hematologic and clinical improvement occurred in each of these patients after crystalline vitamin C was administered.

8. The concept of the multiple deficiency state, with many factors besides vitamin C deficiency adversely affecting the bone marrow, can explain the many conflicting reports on scorbutic anemia which have appeared since 1930.

9. In these eleven consecutive patients the anemia of scurvy occurred primarily because of vitamin C deficiency.

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THE EFFECT OF FOLIC ACID (LACTOBACILLUS CASEI FACTOR) IN NUTRITIONAL HEMATOPENIA OF MONKEYS

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PREVIOUS reports from this and other laboratories¹⁻³ have described the clinical and hematologic aspects of the nutritional deficiency which develops in monkeys when they are maintained on diets deficient in certain constituents of the vitamin B complex. Day and associates^{1-3, 9} have shown that vitamin B deficient diets supplemented with niacin, thiamine, and riboflavin failed to prevent nutritional cytopenia, whereas liver extract or brewers' yeast did prevent these changes. The essential but unidentified component in these two substances was designated vitamin M. Subsequently, in 1942, it was reported from this laboratory⁶⁻⁸ that the addition of calculated supplements of niacin amide, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, paraminobenzoic acid, choline, pimelic acid, and glutamine (Diet 2) failed to prevent the progressive development of the clinical signs of deficiency or the hematopenia, although on this diet leucopenia appeared more consistently in the monkeys in our studies and was usually more pronounced than anemia. Thus, it became evident that the essential component present in liver and yeast was not identical with, or completely included in, these additional individual or collective constituents of the vitamin B complex. Our observations at that time suggested that a folic acid concentrate of yeast was effective in "re-establishing a normal white cell equilibrium" and that this folic acid "closely simulated the effect of parenteral liver extract."^{6, 8} It has since been reported that *Lactobacillus casei* factor has been used effectively in completely compensating for the hypothetical vitamin M deficiency in the monkey.⁹ The purpose of this communication is to report in more detail our studies of the effects of folic acid concentrate of yeast and of crystalline *L. casei* factor from liver in nutritional hematopenia in the monkey.

Following a preliminary period of observation, five monkeys were placed on a synthetic diet and were studied for nutritional changes in the same manner, using the same techniques of feeding as have been previously described.⁶ Two of the animals (Monkeys 53 and 136) received the vitamin B free basic diet, supplemented with all of the separate constituents of the vitamin B complex previously listed (Diet 2), while three animals (Monkeys 61, 1C, and 96) were given the basic diet alone, supplements being added only after signs of nutritional deficiency appeared.

Three of the five animals (Monkeys 53, 61, and 136) were treated intramuscularly with a folic acid concentrate* from yeast¹⁰ containing approximately 57 μ g of *L. casei* factor per cubic centimeter. The other two (Monkeys 96 and

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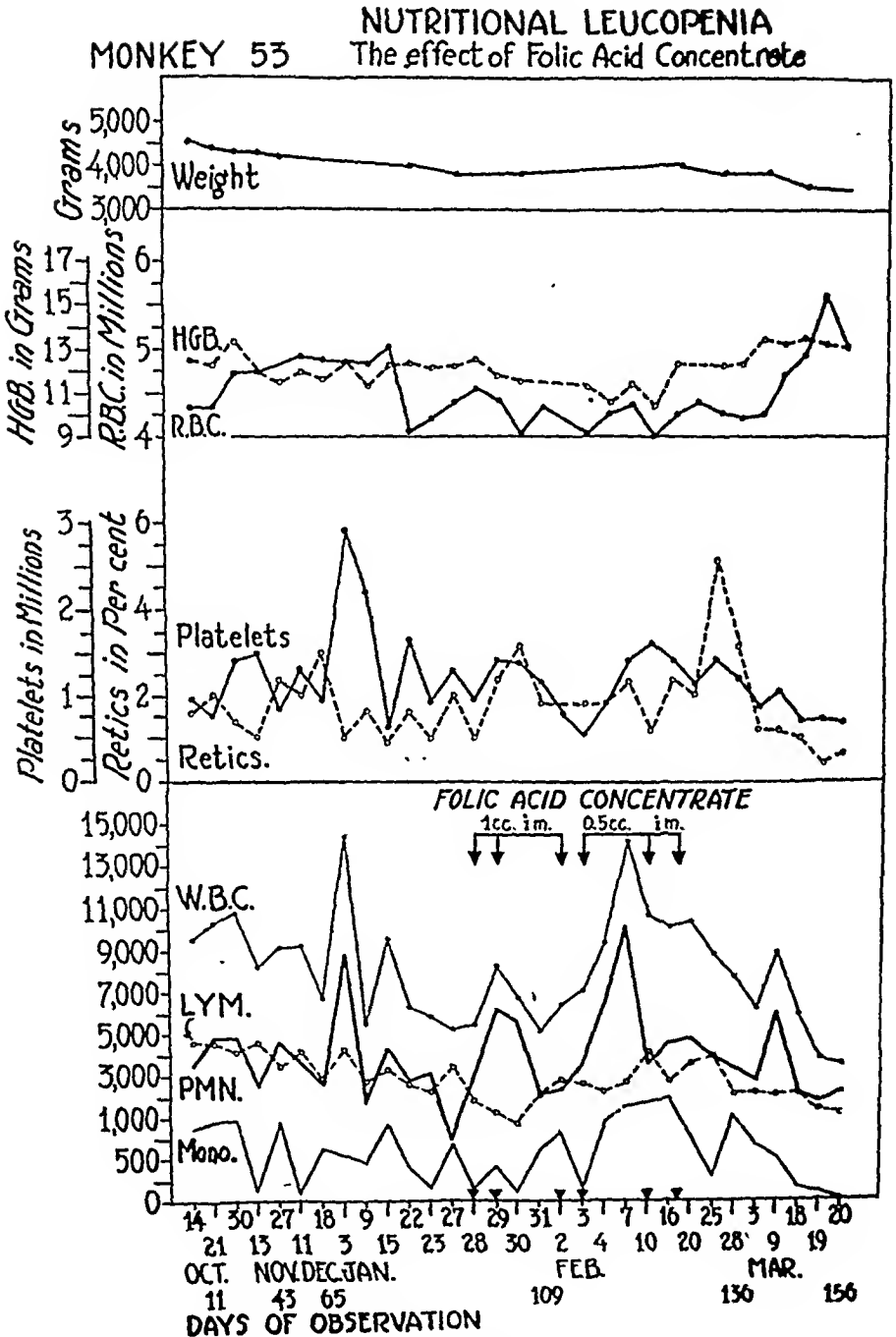


FIG. 1.—This animal on Diet 2 showed a progressive granulopenia and lymphopenia with only a minimal degree of anemia. The initial granulocyte response occurred twenty-four hours and the maximum eleven days after starting folic acid concentrate.

1C) were given a highly purified crystalline preparation of *L. casei* factor¹¹ intravenously.*

Monkey 53 (Fig. 1), while on Diet 2, showed gradual weight loss and later some diarrhea during the first seventy diet days, but the general appearance and vitality of the animal remained fairly good. Despite the wide fluctuations in granulocytes, the total W.B.C., granulocytes, and lymphocytes fell progressively, until by the eighty-eighth day of diet the white cells had fallen from a total of 10,350 with 4,864 neutrophils and 4,243 lymphocytes to 5,150 W.B.C. with only 669 granulocytes and 3,502 lymphocytes.

Intramuscular injections of folic acid concentrate totaling 4.5 c.e. were given in six divided doses over a period of twenty days (Fig. 1). There was a prompt increase in granulocytes which reached a peak of 10,080 (higher than normal base line levels) eleven days after the first injection. Following discontinuance of the folic acid, there was a progressive decline in the total white cells which, however, remained within normal limits for twenty-nine days after the last injection.

There was a minimal degree of anemia at the time the folic acid concentrate was given. The red cells did not fall below 4.0 million, as compared with base line levels of from 4.3 to 5.0 million, while hemoglobin values fluctuated between 11.5 and 12.5 Gm., as compared with base line values of from 13.2 to 14.0 Gm. There was a slight increase in reticulocytes to 3 per cent within three days and a secondary rise to 5.4 per cent twenty-eight days after beginning the folic acid injections. There followed an increase in total red cells to 5,700,000. After this brief period of hematologic and clinical improvement, there was progressive decline and ultimate death of the animal (Monkey 53). In this monkey the granulocytes and lymphocytes were the most sensitive index to the dietary deficiency in an essential nutritional and hematopoietic component, and accordingly they reflected a more immediate, though transitory, response to folic acid supplement than the erythroid elements or the thrombocytes.

Monkey 136 was maintained on Diet 2 for 135 days prior to the experimental studies charted in Fig. 2. During that initial period the animal gradually developed clinical signs of nutritional deficiency, including anemia, leucopenia, and thrombocytopenia. The weight declined from 3,060 to 2,480 grams. The total white cell count fell from a base line average of from 10,200 to 5,000 and the granulocytes from 6,400 to 3,600, and the lymphocytes were reduced from 2,000 to 720 per cubic millimeter. Similarly, the red cells gradually dropped from 4.5 to 5.0 million to a low point of 2.9 million and the hemoglobin from 12.5 to 14.0 Gm. to 7.8 Gm. The platelets fell from 1,000,000 to 200,000 per cubic millimeter. After the signs of nutritional deficiency and panhematopenia were thus well advanced, crude liver extract was administered intramuscularly daily, for eight days, and the animal was returned to a normal diet for thirty days. A prompt reticulocyte crisis followed with a transitory overcompensatory granulocytosis and thrombocyte recovery. Coincident with this hematologic response, there was marked clinical improvement. This monkey,

*We are indebted to Dr. E. L. R. Stokstad for this material.

however, did not regain the weight lost during the first period on Diet 2, and there was a persistent diarrhea. When the animal was again placed on Diet 2, granulocytes, lymphocytes, and erythrocytes fell again promptly. On the fifty-fourth diet day folie acid concentrate injections were begun; they resulted in an immediate significant granulocytosis and a later rise in red cells, hemoglobin,

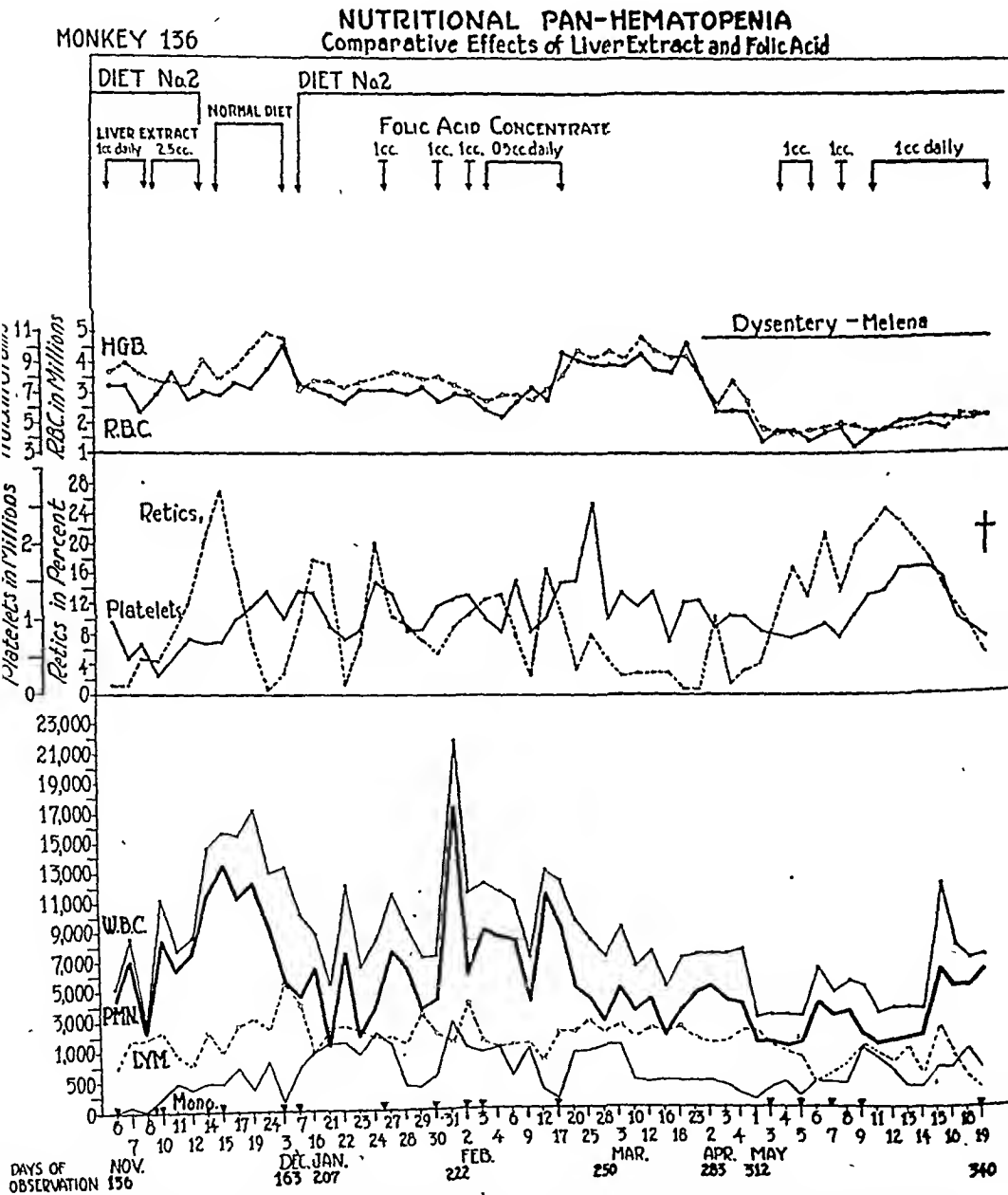


Fig. 2.—The response to the first series of folie acid concentrate injections is strikingly similar to that of liver extract, with an increase in all of the white cell elements, red cells, hemoglobin, and the blood platelets.

and platelets comparable to that noted following the earlier liver extract injections. The first series of daily folic acid concentrate injections was stopped on the seventy-fourth day of diet and was followed by a decline in granulocytes. The clinical state of the animal deteriorated rapidly, and the decline was accelerated by a spontaneous *Shigella dysenteriae* infection. The loss of blood in the feces at this time precipitated a profound anemia. The bone marrow study (April 3, 1942) revealed an absolute hypoplasia of both the erythroid and myeloid elements with a slight left shift in the erythroid elements. In spite of the fact that the animal was in a profound state of debility, another series of folic acid concentrate injections was begun and resulted in a reticulocyte crisis with a peak of 24 per cent and an abortive granulocytosis and thrombocytosis. These reactions were quantitatively less than the earlier marrow responses to liver and folic acid, respectively, and inadequate to initiate recovery from the established intestinal infection. Terminally, a study of the femoral bone marrow revealed a more advanced degree of erythroid and myeloid hypoplasia than had been observed seven weeks earlier.

Monkey 61 (Fig. 3) was maintained on the basic diet, supplemented for the first few days of the experimental period by oral yeast. On this regime diarrhea and leucopenia developed and led to a very rapid decline in the clinical state of the animal. An optimum diet was reinstituted with prompt clinical improvement and a moderate reticulocyte increase and hemoglobin rise but with the white cell levels increased only slightly. The basic diet was then resumed without vitamin B supplements, and by the end of four months a relatively profound anemia had developed.

During this period the animal showed several distinct episodes of clinical decline associated with a further progressive leucopenia, with transitory remissions in the leucopenic phases paralleled by temporary clinical improvement (Fig. 3). On Feb. 24, 1942, the total white blood cells fell to 6,350, as contrasted with base line levels of from 11,000 to 16,800, and the neutrophils fell from a base line of from 6,500 to 1,524 per cubic millimeter. Again on May 19, 1942, the leucocytes fell to 8,500 and the granulocytes to 1,400. By the one hundred twenty-eighth day on this diet there was marked anemia and leucopenia and clinical debility. At this point folic acid concentrate was started. The animal received 2.0 c.c. intramuscularly daily, for eight successive days; then after a lapse of one week the injections were continued, 2.0 c.c. every second day for a period of fifty-four days. After the administration of this material, there was a sharp immediate response in granulocytes, followed shortly by an increase in reticulocytes and platelets. The granulocytes reached a peak of 16,000 with the total white cells 19,250 in an overcompensatory rebound on the eighth day of therapy and then returned to base line levels (from 4,000 to 5,000), coincident with an increase in lymphocytes up to 10,000 per cubic millimeter. A marked increase in thrombocytes to 3,700,000 per cubic millimeter coincided with the maximum reticulocyte response of 25.6 per cent which was observed after nine days. There followed an increase in circulating red cells to 5.5 million from 3.2 million and in hemoglobin to 14.4 Gm. from 8.9 Gm. after folic acid supplements. Following this period of hyperresponse, the animal was returned to an

optimum diet supplemented by parenteral liver extract. The red cells and hemoglobin resumed their normal equilibrium. There was gradual clinical improvement and weight gain over a period of six weeks after beginning folic acid concentrate, but at no time did the animal (Monkey 61), completely lose the hyperkeratosis and scaling of the skin which had appeared during the period of deficiency nor completely regain its normal weight and appearance of well-being. The anemia in this monkey on a limited basic diet was more pronounced

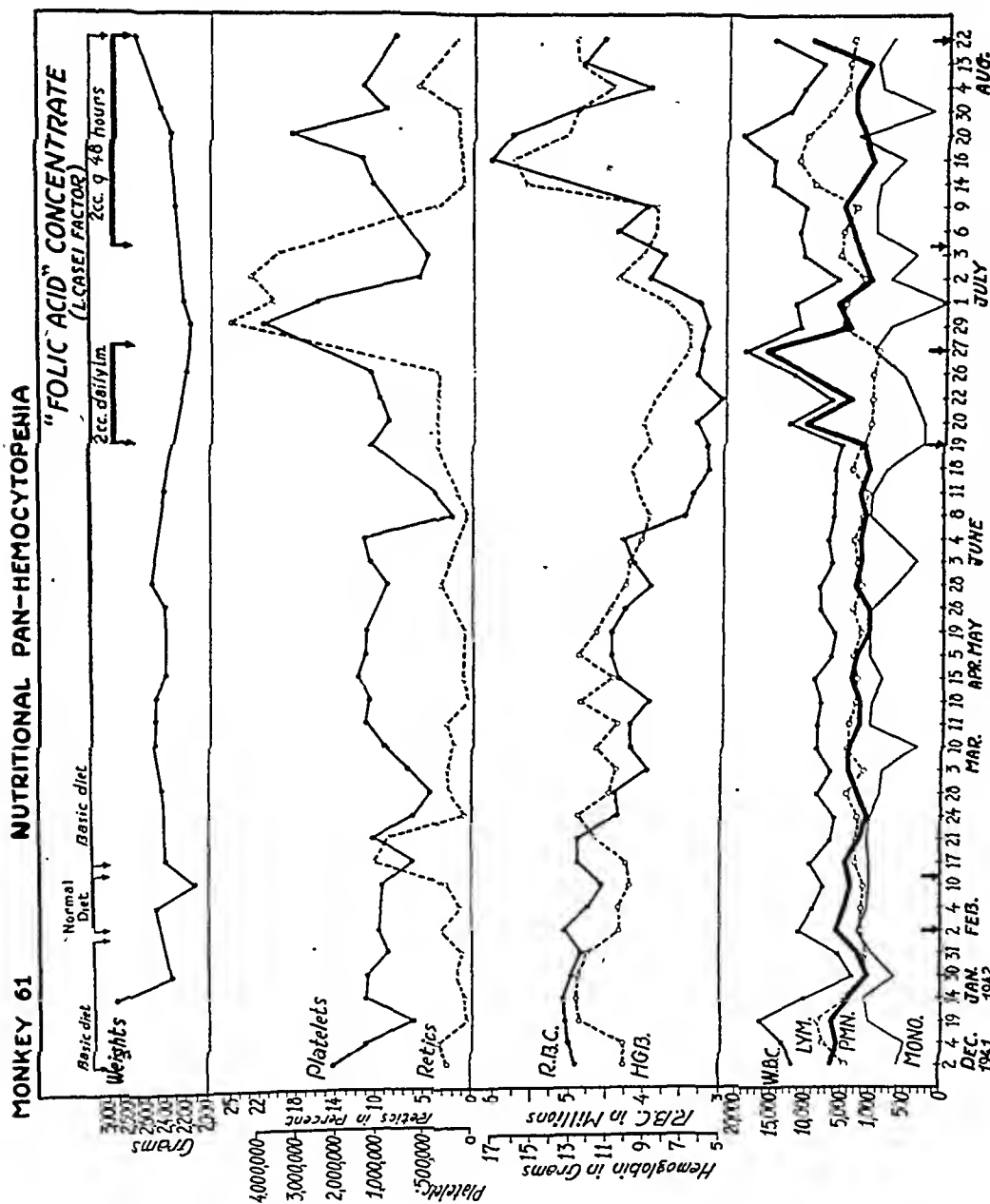


Fig. 3.—The first response to folic acid concentrate was an increase in neutrophils within twenty-four hours, which was soon followed by crises in the reticulocytes and platelets. The increase in lymphocytes, red cells, and hemoglobin paralleled the weight gain and preceded the clinical improvement.

than that seen in most of those animals maintained on the basic diet plus supplemental B fractions, and the response of the erythroid elements to folic acid was correspondingly more dramatic.

COMPARISON OF THE EFFECTS OF ADENYLIC AND FOLIC ACID

The role of adenine and guanine nucleotides in myelopoietic stimulation has been experimentally demonstrated^{12, 13} and the principle applied therapeutically¹⁴⁻¹⁶ in recent years. Because of the apparent specific effect of *L. casei* factor on myelopoiesis in nutritional leucopenia, it seemed desirable to compare the effects of folic acid and adenylic acid under the conditions of this experiment.

Monkeys 96 and 1C were placed on basic diet without the vitamin B supplements in order to produce the deficiency syndrome rapidly. Both animals showed a similar response throughout the parallel periods of observation (Figs. 4 and 5). During a period of sixty-two days on this restricted diet the total white cells in Monkey 96 fell from an initial 18,000 to 21,000 base line to 6,900; the granulocytes, from 16,000 to 2,400; and the lymphocytes, from 3,900 to 1,100. A significant anemia also developed, the red cells falling from 5.5 to 6.0 to 2.88 million per cubic millimeter and the hemoglobin from 12.7 to 7.1 Gm. per 100 c.c.

At this time daily intravenous injections of adenylic acid (0.2 mg.) were started. During this part of the investigation complete blood studies were made four times daily. It was observed that for a few hours after each daily intravenous injection there was a temporary marked leucocytosis. The high granulocyte peaks recorded were obtained during these early postinjection periods. However, the lymphocytes remained abnormally low, and the granulocytes returned to leucopenic levels within from eight to twelve hours after each injection. The red cells, hemoglobin, reticulocytes, and platelets were unaffected.

Monkey 96 then was given daily intravenous injections of *L. casei* factor (from 0.1 to 0.2 mg., see Fig. 4). In response to this substance there was a progressive upward trend in both granulocytes and lymphocytes, with total white cells varying from 9,000 to 16,000 and granulocytes from 6,000 to 10,000, and lymphocytes remaining fairly stable at from 2500 to 3000 per cubic millimeter. There was an appreciable reticulocyte response (6.8 per cent after 0.7 mg. of *L. casei* factor had been administered over a period of twenty-two days, and a secondary reticulocyte peak occurred after further continued daily administration. A slow, but progressive, rise in red cells (from 4.5 to 5.5 million) and hemoglobin (from 12.6 to 13.0 Gm.) took place during this seventy-day period of *L. casei* factor administration. The clinical state of the animal improved coincident with the hematopoietic recovery, and this improvement continued and was maintained after the addition of the other vitamin B supplements. However, the animal was not entirely normal. There was marked irritability and a tendency to jerky spasmodic movements which did not disappear until this monkey had been on a normal diet for a number of weeks after the end of the experiment. Monkey 1C (Fig. 5) showed a somewhat more profound nutritional leucopenia and a less marked temporary leucocytosis in response to adenylic acid administration. However, the cumulative and sus-

NUTRITIONAL PAN-HEMATOPENIA Contrasting Effects of Adenylic and Folic Acid

MONKEY No 96

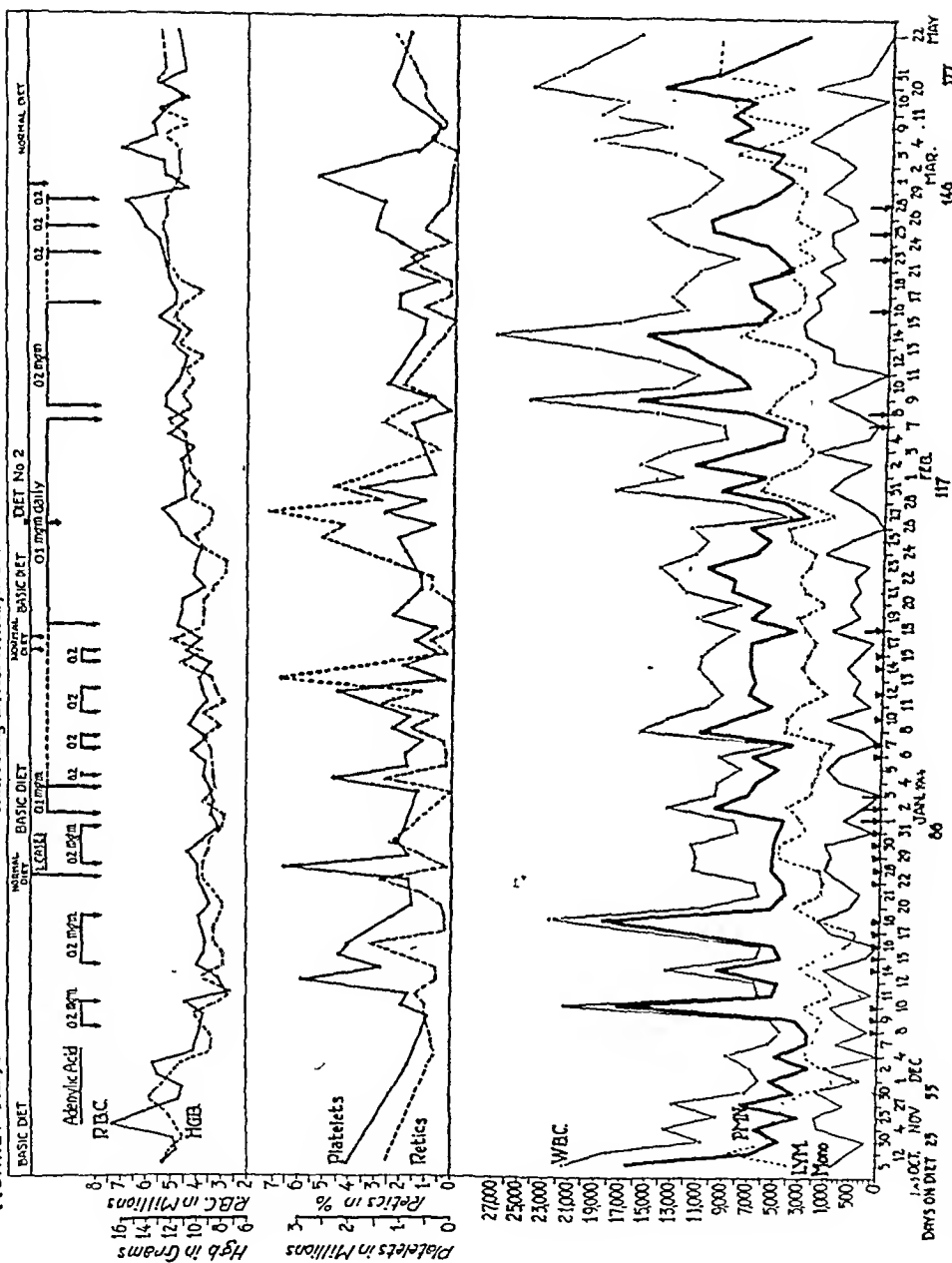


Fig. 4.—The response to each adenylic acid infection was a sharp transitory elevation of the granulocytes followed by a rapid return to leucopenic levels with no appreciable influence on the red cells. The response to L_2 cast factor was a progressive increase in neutrophils and lymphocytes with a low reticulocyte peak followed by recovery of the red cells and hemoglobin. The subsequent addition of the other 13 complex supplements (Diet 2) seemed to augment the initial L_2 cast effect, both clinically and hematologically.

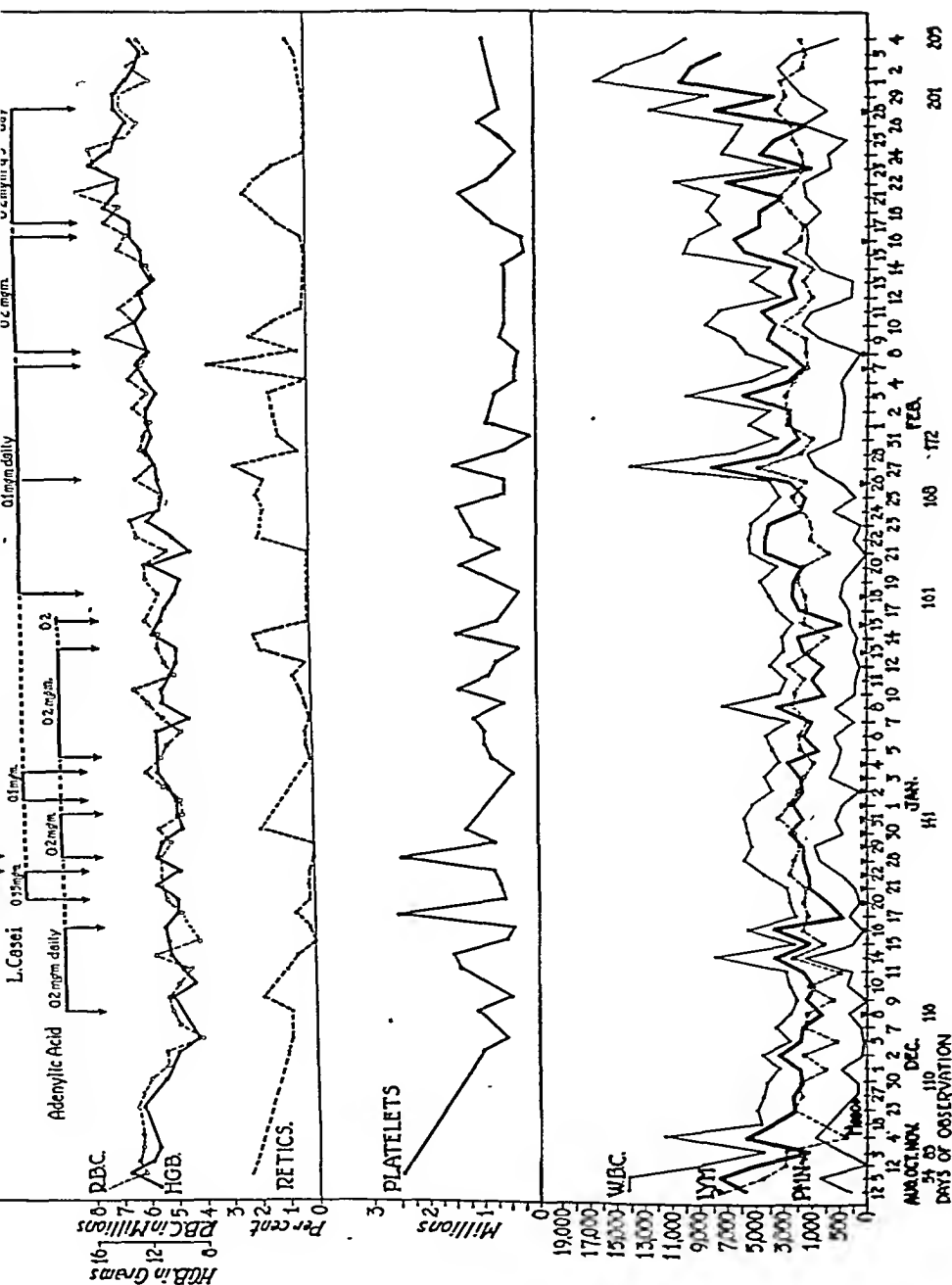


Fig. 5.—The nutritional neutropenia and lymphopenia were more profound in this animal than in Monkey 96 (Fig. 1). Therefore, the progressive recovery of the white cells, including the monocytes, and the steady rise in red cells was correspondingly more apparent and dramatic following the substitution of *L. casei* factor for adenyllic acid.

tained erythroid and myeloid recovery following *L. casei* factor, as contrasted with adenylic acid, was as striking and definite as that in Monkey 96 just described.

In control studies three normal monkeys, each on optimum diet, were given daily intravenous injections of 0.2 mg. of adenylic acid over a period of several weeks. A marked transitory leucocytosis was observed for a few hours after each injection with subsequent return of white cell levels to normal within twelve hours.

Two other normal monkeys in an optimum nutritional state received repeated intravenous injections of 0.2 mg. of synthetic *L. casei* factor with no significant alteration in any of the peripheral blood cell levels.

DISCUSSION

All of the five animals on the experimental diets described herein developed clinical and hematologic evidences of nutritional deficiency. In these animals, as in many others which we have observed on similar diets, the neutropenia developed first. The lymphopenia which appeared independently, later, was less subject to exacerbations and remissions than the neutropenia and more accurately reflected the degree of nutritional and physical deficiency of the animal. The rapidity of development and the degree of the panhematopenic tendency, like the clinical signs, varied with the individual animal. Thus, one animal (Monkey 53) showed only the leucopenia; the other four monkeys showed anemia plus leucopenia; and two of these (Monkeys 61, and 136) developed a significant thrombocytopenia. Individual variation was also observed by Langston and co-workers who reported that "a few animals died before the anemia became marked" and that "the degree of anemia varied somewhat with the animal."

The contrasting effects of adenylic acid, causing a transient leucocytosis, and of *L. casei* factor, which stimulated a gradual sustained increase in both myeloid and erythroid elements, are clearly demonstrated.

It is possible that the folic acid concentrate which was used in three of the animals may have contained minimal components of yeast other than *L. casei* factor. Whatever other substances may have been present, however, were utilized precisely the same as is *L. casei* factor, as indicated by the similar nature and identical character of the hematologic responses.

The report of Day and associates⁹ indicates that the addition of purified *L. casei* factor to similarly supplemented basic diets constitutes an adequate diet for the monkey. While this substance in the quantities administered to the monkeys in our studies was not entirely effective in returning the animals to a completely normal nutritional state within the period of these observations, there was restoration of the bone marrow to normal function with the re-establishment of a normal equilibrium of the cells in the peripheral blood, associated with marked clinical improvement.

It is likely that the anorexia shown by monkeys in the well-defined vitamin M deficiency state results in decreased diet consumption and consequently a rela-

tive protein deficiency which may play some part in the clinical and hematologic pictures. Similarly, it is possible that a dietary deficiency in *L. casei* factor or substances utilizable as *L. casei* factor may precipitate a relative insufficiency in other components of the vitamin B complex, despite the addition of these to the basic diet. Whatever role these factors may play, it seems clear that the *L. casei* factor is an essential molecule for normal hematopoiesis and optimum nutrition in the monkey. Furthermore, it seems that the potential activity of *L. casei* factor comprises not only an essential antianemia element but also a very potent myelopoietic and thrombocytopoietic stimulus for optimum bone marrow function. The critical deficiency threshold in any given animal at any given time may differ for red blood cells, granulocytes, lymphocytes, and blood platelets. The hematologic and clinical manifestations which result from a diet lacking the essential hematopoietic elements reflect the degree of inadequacy of the vital raw materials and/or catalysts necessary for erythroid, myeloid, lymphoid, and megakaryocytic functions, respectively.

Other recent studies in monkeys¹⁷ and clinical studies in human nutritional leucopenia¹⁸ and in E.M.F. deficiency anemias¹⁹⁻²² using synthetic *L. casei* factor²³ lend support to this concept of *L. casei* factor as an essential pan-hematopoietic substance.

SUMMARY

1. Five monkeys (*Macaca mulatta*) on vitamin B deficient basic diets supplemented in four cases by all of the known B vitamin fractions except *L. casei* factor developed clinical and hematologic evidences of nutritional deficiency.

2. Three of these animals treated with a yeast autolysate containing *L. casei* factor showed dramatic clinical remissions and the re-establishment of a normal hematopoietic equilibrium.

3. Two of these animals treated with purified crystalline *L. casei* factor from liver showed unequivocal clinical and panhematopoietic remissions after failure of adenylic acid to evoke more than a very transitory, nonecumulative, or sustained leucocytosis.

4. These studies indicate that a monkey under the conditions of these observations may develop a deficiency in one or all of the bone marrow elements, any one or all of which may respond promptly and completely to *L. casei* factor activity.

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FURTHER OBSERVATIONS ON THE ANTIANEMIC PROPERTIES OF 5-METHYL URACIL

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FOR many years the subject of macrocytic hyperchromic anemia has been under intensive investigation in many laboratories and clinics. The relationship of nutrition to the formation of blood cells has been one of the many divergent lines of these investigations. Since 1940 we have been interested in the function of thymine^{*} in this relationship, since it is a normal constituent of the body cell. We observed that the administration of 1 Gm. or less of 5-methyl uracil gave no hematologic response when given to patients with nutritional macrocytic anemia, the anemia of sprue, and pernicious anemia in relapse.¹ At a higher dose level a hematologic response was observed in patients with pernicious anemia in relapse.² The present report is concerned with the effectiveness of synthetic 5-methyl uracil as a hemopoietic substance.

The criteria used in the selection of the patients were as follows:

1. An erythrocyte level of 2.5 million or less
2. A color index of 1.0 or more
3. A mean corpuscular volume of 100.0 cubic microns or greater
4. A mean corpuscular hemoglobin of 32 micromicrograms or more
5. A mean corpuscular hemoglobin concentration of 34 per cent or more
6. A histamine refractory achlorhydria and achylia
7. A normal oral glucose tolerance curve
8. The absence of acute infections

Using these criteria, we selected six patients for study. A complete dietary and medical history was obtained, and a physical examination was performed. Gastrointestinal x-rays, urinalyses, and stool examinations were essentially normal in all instances, and an icteric index obtained prior to treatment was slightly elevated in each case. Sternal bone marrow studies performed on each patient before treatment was instituted revealed the megaloblastic arrest characteristic of E. M. F.† deficiency anemia.

Each patient selected for study was hospitalized, and base line laboratory studies were done. The diet was rigidly controlled and contained no meat, meat products, fish, poultry, or raw vegetables. Blood studies were performed

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*Because of the confusion arising from the similarity in pronunciation between thymine (5-methyl uracil) and thiamine (vitamin B₁), we suggest that the name 5-methyl uracil be employed when referring to the former compound, and we shall use this term in the present publication.

†Erythrocyte maturation factor.

TABLE I. ORAL DOSAGE OF SYNTHETIC 5-METHYL URACIL ADMINISTERED TO SIX PATIENTS

CASE	COURSE OF TREATMENT	DAILY DOSE (GM.)	NUMBER OF DAYS	TOTAL DOSE (GM.)	REMARKS
1	1	1.0	7	7.0	No therapy given for period of 45 days between Courses 2 and 3
	2	6.0	12	72.0	
	3	10.0	11	110.0	
2	1	10.2	11	112.2	No therapy given for period of 17 days between Courses 1 and 2
	2	10.0	13	130.0	
3	1	4.5	13	58.5	No therapy given for period of 5 days between Courses 2 and 3
	2	10.2	5	51.0	
	3	10.0	11	110.0	
4	1	6.0	14	84.0	
5	1	12.0	8	96.0	
6	1	6.0	12	72.0	

daily as previously described.³ Bone marrow studies on each patient were performed prior to treatment, on the second day after the peak reticulocytosis had occurred during treatment, and again after the reticulocyte concentration had subsided to less than 3 per cent in the peripheral blood. Immediately prior to administration 5-methyl uracil was weighed on an analytic balance and suspended in one-half glass of water. The doses administered varied in each case, as shown in Table I.

TABLE II. EFFECT OF ORAL ADMINISTRATION OF SYNTHETIC 5-METHYL URACIL ON PERIPHERAL BLOOD IN PERNICIOUS ANEMIA IN RELAPSE

CASE	COURSE OF TREATMENT	R.B.C. (MILLION)			HB. (GM.)			RETIC. (%)			TOTAL DOSAGE (GM.)
		INITIAL	AFTER 7 DAYS	FINAL DAY	INITIAL	AFTER 7 DAYS	FINAL DAY	FIRST DAY OF RISE	DAY OF PEAK	AT PEAK	
1	1	1.99	1.73	2.01 (8)	7.5	7.0	6.3 (8)	-	-	-	7.0
	2	2.01	2.19	2.88 (22)	6.3	8.1	10.4 (22)	6	11	14.2	72.0
	3	2.37	2.48	2.98 (11)	8.0	9.0	10.2 (11)	4	10	13.2	110.0
2	1	1.58	2.02	2.51 (17)	6.2	8.4	9.8 (17)	4	6	19.0	112.2
	2	2.25	2.33	2.74 (13)	9.8	9.0	10.4 (13)	5	8	14.2	130.0
3	1	1.68	1.92	2.01 (13)	7.5	8.6	9.0 (13)	6	11	9.0	58.5
	2	1.93	2.55	2.55 (7)	9.4	10.0	10.9 (7)	5	6	8.8	51.0
	3	2.38	2.28	2.70 (13)	10.4	10.3	10.7 (13)	6	9	5.4	110.0
4	1	1.86	2.02	2.34 (14)	5.8	6.0	7.5 (14)	4	11	16.0	84.0
5	1	1.34	1.61	1.60 (8)	5.4	6.6	6.8 (8)	3	7	31.2	96.0
6	1	2.22	2.55	2.79 (12)	10.7	11.3	12.0 (12)	2(?)	5	14.4	72.0

The hematologic effect of 5-methyl uracil was observed in six patients with pernicious anemia in relapse, as shown in Table II. Coincident with the reticulo-
cyte response, there was in each case a definite upsurge in well-being, an improve-
ment in appetite, and an increase in the patient's interest in his surroundings.
The subjective and hematologic response to the oral administration of 5-methyl
uracil was in every way similar to that following the administration of folic acid
to patients with pernicious anemia in relapse.

Case 1. Reduplication of the Hematologic Response in the Same Patient to Large Doses of 5-Methyl Uracil.—This patient was given 500 mg. of 5-methyl
uracil b.i.d. orally for a period of seven days without hematologic response.

Age: 59 years. Col. ♀ Histamine Refractory Achlorhydria and Achylia

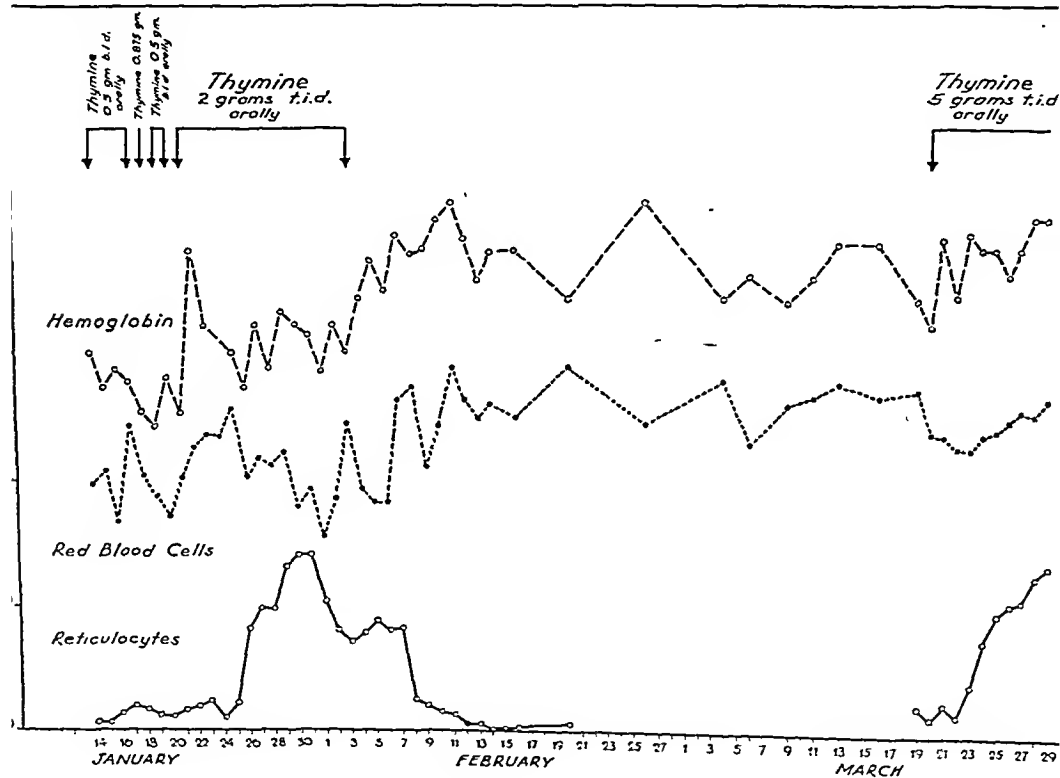


Fig. 1.

The dose was then increased to 2 Gm. t.i.d. On the sixth day after treatment with the larger dose had been instituted, the reticulocytes increased to 8.2 per cent, and a peak reticulocytosis of 14.2 per cent was reached on the eleventh day. Treatment was discontinued after twelve days, but the erythrocytes and hemoglobin continued to rise to a level of 2.88 million and 10.4 Gm. (68 per cent), respectively, on the twenty-second day after treatment with the larger dose was instituted. This was a net increase in erythrocytes of 870,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 4.1 Gm.

within a period of twenty-two days. Sternal bone marrow preparations made at this time showed a reversion toward normal. The patient was then discharged from the hospital and subsequently was observed on several occasions in the Nutrition Clinic. Blood studies were performed biweekly, and it was observed that the erythrocyte level and hemoglobin remained essentially constant until the week prior to readmission to the hospital.

Forty-five days after the patient had received her last dose of 5-methyl uracil, she was readmitted to the hospital for further study. In an effort to reproduce the hematologic response we had initially observed after administration of large doses of 5-methyl uracil in this patient, we resumed treatment, giving the patient 5 Gm. of 5-methyl uracil b.i.d. orally. A reticulocytosis of 4.0 per cent occurred on the fourth day of therapy, and a peak reticulocytosis of 13.2 per cent was reached on the tenth day after treatment had been resumed. On the eleventh day after the present therapy had been instituted, there had been a total increase in erythrocytes of 610,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 2.2 Gm. These results are shown graphically in Fig. 1.

Case 2. Production of a Maximal Hematologic Response to 5-Methyl Uracil.—In an effort to produce the expected maximal hematologic response as calculated by Minot and associates,⁴ this patient was given larger doses of 5-methyl uracil than Case 1 received initially. Such a response was obtained. The patient received 3.4 Gm. of 5-methyl uracil t.i.d. orally for eleven days. On the fourth day of therapy the reticulocyte concentration was 6.0 per cent, and a peak reticulocytosis of 19.0 per cent was reached on the sixth day of treatment. On the seventeenth day after therapy was instituted there had been a total increase in erythrocytes of 930,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 3.6 Gm. Sternal bone marrow obtained on the seventh day of therapy showed regeneration as evidenced by a marked increase in normoblasts and a reduction of megaloblasts. Treatment was discontinued for a period of seventeen days and then resumed, the patient receiving thereafter 5 Gm. of 5-methyl uracil b.i.d. On the fifth day of this second course of treatment the reticulocytes were 5.8 per cent, and a peak reticulocytosis of 14.2 per cent was attained on the eighth day after treatment was instituted. By the thirteenth day the erythrocytes had increased by 490,000 per cubic centimeter of peripheral blood, while the hemoglobin remained essentially unchanged. Sternal bone marrow preparations made at the end of the period of therapy showed a reversion toward normal.

Case 3. Determination of a Minimal Dose of 5-Methyl Uracil Which Will Give a Hematologic Response.—In an effort to determine the minimal dose of 5-methyl uracil necessary to effect a hematologic response within a period of two weeks, and in order to determine the minimal dose required for a full hematologic response by employing the double reticulocyte response described by Minot and Castle,⁵ this patient was given 1.5 Gm. of 5-methyl uracil t.i.d. orally for a total of thirteen days. A reticulocyte response which appeared to be submaximal occurred, as shown in Table II. Sternal bone marrow obtained on the twelfth day of treatment showed evidence of blood regeneration as well as some megaloblasts.

blastic arrest. This was interpreted as being indicative of a response to inadequate dosage. After the reticulocyte concentration began to fall, the dosage of 5-methyl uracil was increased to 3.4 Gm. t.i.d. orally for a period of five days. A second reticulocyte response occurred, a peak of 8.8 per cent being reached on the sixth day after treatment with this larger dose was instituted. On the twentieth day after therapy with 1.5 Gm. of 5-methyl uracil t.i.d. was instituted, there had been an increase in erythrocytes of 620,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 3.4 Gm. Sternal bone marrow obtained at this time was normoblastic and in the phase of active regeneration. The patient received no medication for a period of five days following therapy with the larger dose, and he was then given 5 Gm. of 5-methyl uracil b.i.d. as a maintenance dose. A slight but significant reticulocyte response occurred after treatment was resumed. Thus, it is seen that an oral dose of 5-methyl uracil as small as 4.5 Gm. daily will effect a hematologic response in a patient with pernicious anemia in relapse. It is further observed that 4.5 Gm. of 5-methyl uracil daily is an inadequate dose as evidenced by the subsequent reticulocyte response to 10.2 Gm. of 5-methyl uracil daily and also by the presence of an abnormal number of megaloblasts seen in the sternal bone marrow preparation obtained at the end of the course of treatment with this smaller dose.

J.B. Age 46 yrs. White & Histamine Refractory Achlorhydria and Achylia.

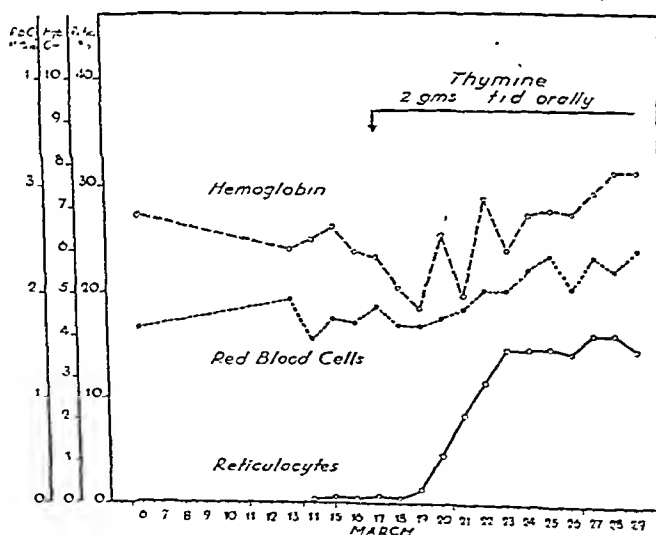


Fig. 2.

Case 4. Hematologic Response to a Suboptimal Dose of 5-Methyl Uracil.—

In an effort to determine the minimal dose of 5-methyl uracil required to produce the full calculated hematologic response, we gave this patient what we now believe to be a suboptimal dose. Two grams of 5-methyl uracil were given orally t.i.d. for a period of fourteen days. On the fourth day of treatment the reticulocyte concentration was 4.6 per cent, and a peak reticulocytosis of 16 per cent

was reached on the eleventh day of treatment. The blood findings in this patient are shown in Fig. 2. Sternal bone marrow obtained on the fourteenth day of treatment was normoblastic and without megaloblasts. It is thus seen that a daily oral dose of 2 Gm. of 5-methyl uracil t.i.d. will effect a profound, although not a maximal, hematologic response when given to a patient with pernicious anemia in relapse. Further studies are now being conducted on this patient in an effort to determine the minimal dose of 5-methyl uracil required for a calculated maximal hematologic response.

Case 5. The Hematologic Response to a Large Dose of 5-Methyl Uracil.—This patient received 4 Gm. of 5-methyl uracil t.i.d. orally for a period of eight days. On the third day of treatment the reticulocytes were 3.6 per cent, and a peak reticulocytosis of 31.2 per cent occurred on the seventh day of treatment. Sternal bone marrow obtained after treatment showed great numbers of normoblasts and a substantial reduction in the number of megaloblasts which was indicative of response to an adequate therapeutic dose.

Case 6. Response to 6 Gm. of 5-Methyl Uracil Given Daily.—This patient had a reticulocyte level of from 3.0 to 4.0 per cent over a period of six days during base line observations. Because of the constancy of this reticulocyte level, we felt that it was normal in this individual and that we were not dealing with a so-called spontaneous remission. Sternal bone marrow preparation studies prior to treatment showed megaloblastic arrest and no evidence of regeneration. The patient received 2 Gm. of 5-methyl uracil t.i.d. orally for a period of twelve days. On the second day of treatment the reticulocytes had reached a level of 5.8 per cent, and a peak reticulocytosis of 14.4 per cent occurred on the fifth day of treatment. Sternal bone marrow obtained after treatment was normoblastic and without megaloblasts. It is probably incorrect to state that this patient responded to treatment as early as the second day. However, we believe it is correct to say that this patient responded hematologically to large doses of 5-methyl uracil.

DISCUSSION

The scientific investigation of pernicious anemia dates from the time of Thomas Addison when, in 1855, he described the anemia which now bears his name. A milestone in these investigations was passed in 1926, when Minot and Murphy⁶ effected a hematologic remission in patients with pernicious anemia by feeding them large amounts of liver by mouth. Not until the fall of 1945, when synthetic folic acid was shown to have antianemia effects in patients with macrocytic hyperchromic anemia,^{3, 7} were investigators able to use pure synthetic compounds in their studies. The studies on folic acid have been extended, and the efficacy of this substance in the treatment of pernicious anemia, nutritional macrocytic anemia, and the anemia of sprue has been established.⁸⁻¹² The observation that 5-methyl uracil will produce a hematologic remission in patients with pernicious anemia in relapse necessitates further studies of the functional relationship between folic acid and 5-methyl uracil. We realize that folic acid may act as an enzyme or as a coenzyme in the synthesis of 5-methyl uracil or of substances which act similarly. Studies are likewise indicated as to the efficacy

of synthetic 5-methyl uracil in the treatment of nutritional macrocytic anemia and the anemia of sprue. This compound will probably prove as effective in the treatment of these two types of macrocytic hyperchromic anemia as in the treatment of pernicious anemia in relapse.

SUMMARY AND CONCLUSION

Six patients with Addisonian pernicious anemia in relapse were selected for study. They were hospitalized and given a diet devoid of meat, meat products, fish, poultry, and raw vegetables. After an initial period of base line studies, synthetic 5-methyl uracil was administered in varying doses by mouth to each patient. Six patients are reported in whom a hematologic response was produced by the daily oral administration of synthetic 5-methyl uracil when given in doses of 4.5 Gm. or more. Case 1 is of special interest in that the hematologic response to large doses of 5-methyl uracil was reduplicated after a period of forty-five days in which no therapy was given. Case 2 shows the production of calculated maximal hematologic response after the daily oral administration of 10.2 Gm. of 5-methyl uracil.

It is concluded that synthetic 5-methyl uracil is a potent antianemic substance when given orally in daily doses of 4.5 Gm. or more to patients with pernicious anemia in relapse.

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EFFECT OF PENICILLIN AND SULFONAMIDES ON ACETYLCHOLINE SYNTHESIS AND ON THE RESPONSE OF MUSCLE TO ACETYLCHOLINE AND POTASSIUM*

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A RECENTLY presented concept concerning the pathogenesis of myasthenia gravis suggests that there is a defect in either the activity or the amount of enzyme that synthesizes acetylcholine.¹⁻³ Since patients with myasthenia gravis may in addition suffer from infectious diseases, some of which are treated with penicillin and sulfonamides, it becomes pertinent to investigate the effect of penicillin and sulfonamides on acetylcholine synthesis.

EXPERIMENTAL

Effect of the Substances on Acetylcholine Synthesis.—The effect of penicillin and sulfonamides on the acetylcholine synthesis was studied by a method described previously.³ Mixtures containing varying amounts of these drugs, 100 mg. of minced fresh frog brain, 3 mg. of physostigmine salicylate, and 3 c.c. of Ringer's solution, were shaken and incubated aerobically for four hours at 37° C. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog.

Calculation: The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical nonincubated mixtures. The amount of acetylcholine synthesized in the control mixtures containing only brain, physostigmine, and Ringer's solution was taken as 100 per cent. The acetylcholine content of the mixtures containing penicillin and sulfonamides was expressed as a per cent of the control.

The results are given in Table I. The sulfonamides studied did not modify the acetylcholine synthesis. Penicillin, however, even in relatively low concentrations, decreased the acetylcholine synthesis.⁴

Effect of the Substances on the Response of Striated Muscle to Acetylcholine.—Acetylcholine participates in the humoral transmission of the effects of stimulation of the motor nerve to the striated muscle. There are many substances known to increase the response of the muscle to acetylcholine, and many of them act by inhibiting the hydrolysis of acetylcholine by decreasing the activity of cholinesterase.

In the following experiments the effect of penicillin and sulfonamides on the response of the muscle to acetylcholine was investigated, following a method described previously.⁵ The rectus abdominis muscle of the frog was excised

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TABLE I. EFFECT OF SULFONAMIDES AND PENICILLIN ON ACETYLCHOLINE SYNTHESIS

SUBSTANCE	AMOUNT OF ACETYLCHOLINE SYNTHESIZED IN PER CENT OF CONTROL*									
	<i>Amount of Sulfonamides (mg.) Added to 100 mg. Frog Brain</i>									
	3	0.3	0.03	0.003						
Sulfanilamide	113	117	114	107						
Sulfapyridine	109	106	104	105						
Sulfathiazole	100	106	107	104						
Sulfadiazine	99	100	96	97						
	<i>Amount of Penicillin (Oxford Units) Added to 100 mg. Frog Brain</i>									
	30,000	15,000	3,000	1,500	300	150	30	15	3	
Penicillin	6	15	35	50	68	90	92	94	99	

*Each value represents the average of ten separate experiments. The standard error of the mean for each value was less than ± 5 per cent.

and suspended in a muscle chamber containing 10 c.c. of Ringer's solution. Shortening of the muscle was induced by immersion in a Ringer's solution containing 50 μ g acetylcholine bromide per 100 c.c. for two minutes. The shortening of the muscle was registered by an isotonic lever on a kymograph. The muscle was then washed with Ringer's solution for ten minutes and shortened again with the acetylcholine solution. This procedure was repeated until three successive immersions in the acetylcholine solution gave similar responses. Then between two shortenings induced by the acetylcholine solution, instead of washing in Ringer's solution for ten minutes, the muscle was washed for only five minutes and immersed in a series of Ringer's solutions containing either penicillin or one of the sulfonamides, in increasing concentrations, for five minutes. The muscle shortening after immersion in the solutions of the drugs was expressed as percentage of the shortening of the same muscle before immersion in the solutions of the drugs. The shortening induced by acetylcholine in control muscles immersed only in Ringer's solution remained unchanged for at least three hours. This period of time was longer than the duration of the experiments described. Since the standard error of the mean for each experiment was less than ± 4 per cent, all results deviating from 100 per cent by more than 12 per cent are probably significant deviations:

$$(2\sqrt{S.E.^2 \text{ (control)} + S.E.^2 \text{ (experiments)}} = 2\sqrt{4^2 + 4^2} = 11.5)$$

The results are given in Table II. Penicillin, in the concentrations used, did not modify the response of the muscle to acetylcholine; sulfonamides, in relatively high concentrations, slightly increased the response of the muscle to acetylcholine.

Effect of Penicillin and Sulfonamides on the Response of Striated Muscle to Potassium.—To ascertain whether the drugs used modify the response of the striated muscle to chemical stimuli other than acetylcholine, the effect of the drugs on the response of the muscle to potassium was also investigated. Potassium was chosen to induce shortening of the muscle, since the effect of potassium usually parallels the effect of direct stimulation of the muscle.^{6, 7}

The rectus abdominis muscle of the frog was prepared as previously described. Muscle shortening was induced by immersion in a 20 mM KCl solution instead of an acetylcholine solution. The effect of penicillin and sulfonamides

TABLE II. EFFECT OF SULFONAMIDES AND PENICILLIN ON CONTRACTION OF RECTUS ABDOMINIS MUSCLE INDUCED BY ACETYLCHOLINE AND POTASSIUM

SUBSTANCE	MAGNITUDE OF CONTRACTION IN PER CENT OF CONTROL;* CONTRACTION INDUCED WITH											
	ACETYLCHOLINE						POTASSIUM					
	Concentration (Mg.) of the Sulfonamides Per 100 c.c. Ringer's Solution											
	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Sulfanilamide	113	102	100	99	100	101	63	86	108	101	100	100
Sulfapyridine	123	102	102	99	98	101	66	79	102	102	100	99
Sulfathiazole	139	111	100	103	101	102	50	76	93	100	102	100
Sulfadiazine	116	100	98	97	99	100	69	78	100	99	99	100
Concentration (Oxford Units) of Penicillin in 100 c.c. Ringer's Solution												
	200,000	100,000	20,000	10,000	1,000	100	200,000	100,000	20,000	10,000	2,000	1,000
Penicillin	96	94	95	98	98	99	230	195	145	138	120	115

*Each value represents the average of ten separate experiments. The standard error of the mean for each value was less than ± 4 per cent.

was ascertained by immersion of the muscle in a series of solutions containing the drugs in increasing concentrations, for five minutes, before immersion in the KCl solution, for two minutes.

The results are given in Table II. Penicillin, in higher concentrations, increased the potassium sensitivity of the muscle, and the sulfonamides, in higher concentrations, decreased the response of the muscle to potassium.

DISCUSSION

Penicillin decreased the amount of acetylcholine synthesized probably because it reduces the activity of the -SH group,⁸ an active group contained in the enzyme involved in the synthesis of acetylcholine.⁹

Since penicillin did not modify the response of the muscle to acetylcholine, it is probable that penicillin, in the concentrations used, does not inhibit cholinesterase. The effect of penicillin in increasing the potassium sensitivity of the muscle suggests that penicillin may sensitize the muscle to direct stimulation.

The sulfonamides, in the concentrations used, did not modify the amount of acetylcholine synthesized. In relatively high concentrations, sulfonamides increased the response of the muscle to acetylcholine, suggesting that they may inhibit cholinesterase in high concentrations. Sulfonamides apparently do not increase the sensitivity of the muscle to direct stimulation.

SUMMARY

1. The effect of penicillin and sulfonamides on acetylcholine synthesis and on the response of the striated muscle to chemical stimuli were investigated.

2. Penicillin, in relatively low concentrations, decreased the acetylcholine synthesis. The sulfonamides did not modify the synthesis of acetylcholine.

3. In the concentrations used, penicillin did not increase the acetylcholine sensitivity of the muscle. Sulfonamides, in higher concentrations, slightly increased the acetylcholine sensitivity of the striated muscle.

4. Penicillin increased the potassium sensitivity of the muscle suggesting that it may render effector cells hypersensitive to direct stimulation. Sulfonamides, in higher concentrations, decreased the potassium sensitivity of the striated muscle.

The authors wish to express their gratitude to E. R. Squibb & Sons, New Brunswick, N. J., for the generous supply of penicillin.

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THE ANTISHOCK ACTION OF ETHANOL IN BURNED MICE

EFFECT ON EDEMA FORMATION AND CAPILLARY ATONY

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IN PREVIOUS reports from this laboratory, it was shown that the administration of 15-unit liver extract increased survival time and diminished mortality in rats and mice subjected to a standardized shock-producing scalding procedure.^{1, 2} The extract was found to be effective if administered prior to scalding, an observation which has been confirmed by Materius and Glasco,³ but not if given after the burn.

During the testing of various fractions derived from 15-unit liver extract, it was observed that one particular fraction, an ethanol extract, was especially active in counteracting shock.⁴ In an effort to purify this fraction, which contained 25 Gm. of ethanol per 100 c.c., it was found that the distillate carried all the antishock activity, whereas none was exhibited by the residue. When, however, ethanol-free 15-unit liver extract was distilled, the residue retained all the activity and the distillate was inactive. In this manner it was shown that the ethanol containing distillate of the special fraction had antishock activity similar to that of ethanol-free liver extract. This led to a study of whether ethanol itself had a preventive effect upon the development of burn shock.

In recent experiments in this laboratory,⁴ it was found that two separate factors were operative in the causation of scald shock, in each instance by reducing the circulating blood volume; the one, local fluid loss, considered by many to play a major role in the initiation of burn shock⁵ and the other, capillary atony, leading to stagnation of blood in the visceral organs.⁶ In scalded animals, it was found that the amount of blood retained in the visceral capillaries was significantly greater than in controls. By experiment it was ascertained that the most satisfactory method of demonstrating blood retention was to make comparative counts of open capillaries and estimations of the hemoglobin content of the viscera, but only after exsanguinating the completely anesthetized animals. In shock, removal from the active circulation of large amounts of blood trapped in atonic vessels in the capillary bed contributes to the observed reduction in the circulatory volume. The capillary atony is believed to be caused by a toxic factor which was demonstrated in the blood of scalded animals by transfusion⁴ and renal perfusion⁷ experiments.

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The relative importance of local fluid loss and the capillary toxic factor in reducing the circulatory volume and initiating shock varied with the character of the burn and the time interval after thermal trauma.

The purpose of this communication is (1) to bring forward evidence that ethanol, like 15-unit liver extract, increases survival time and diminishes mortality in mice subjected to scald shock and (2) to report studies on the mechanism whereby ethanol retards the development of shock.

METHODS

All experiments were performed on Swiss mice weighing from 16 to 26 grams. For any particular experiment, the mice obtained from a single source were of a uniform size. The method of scalding and the details of evaluating results have been reported in a previous communication.² Ethanol in various concentrations was injected intraperitoneally in 0.2 c.c. of physiologic salt solution, an amount which has no measurable antishock activity.² The bleeding volume was ascertained by cutting out the heart of the anesthetized animal and mopping up the blood from the thoracic cavity with a weighed cotton pledget. The amount of fluid loss into scalded limbs was determined by a modification of Blalock's bisection technique.³ All the animals used in these experiments were completely anesthetized with ether immediately prior to scalding. The method of estimating hemoglobin retention in the tissues is given under the appropriate experiment.

Effect of Ethanol on Survival Time and Mortality in Burn Shock.—Twelve groups of at least twenty mice each were injected intraperitoneally with 25 per cent ethanol (0.2 c.c. per mouse) from thirty to forty minutes before scalding to the head at 65° C. for ten seconds. The responses to the thermal injury were compared with those in twelve parallel groups injected with 0.2 c.c. of isotonic salt solution. As shown in Table I, Experiment C, ethanol significantly prolonged survival time and lessened mortality in scalded mice.

The effect of concentration of ethanol was investigated in the same manner. It was found that 0.2 c.c. of 15 per cent ethanol produced a significant increase in survival time and a decrease in mortality, but the effect was not quite as great as that obtained with a 25 per cent solution. The injection of 0.2 c.c. of 5 per cent ethanol was without measurable activity. The results are presented in Table I, Experiments C, D, and E.

These experiments show that ethanol administered prior to scalding displays antishock activity similar to that noted in previous studies with ethanol-free 15-unit liver extract.^{1, 2} That the observed effects on survival time and mortality rate are not due to an intraperitoneal injection itself is proved by the negative results obtained in control experiments with 0.2 c.c. of isotonic salt solution injected intraperitoneally and with a variety of other substances tested in previous studies.³ To ascertain the effect of a peritoneal irritant, tincture of catharides was injected intraperitoneally into mice prior to scalding. The results of this experiment were negative.

Effect of Time of Injection on Antishock Activity of Ethanol.—In Table I, data are presented on the protective activity of 0.2 c.c. of 25 per cent ethanol

TABLE I. EFFECT OF ETHANOL ON SURVIVAL TIME AND MORTALITY IN BURN SHOCK

EX- PERI- MENT	PER CENT ETHANOL (GM. PER 100 GM.)	TIME OF IN- JECTION IN RELATION TO BURN	NUM- BER OF TRIALS	TOTAL NUM- BER OF MICE	PER CENT INCREASE IN AVERAGE SURVIVAL TIME OVER CONTROLS		P† VALUE	INCREASE IN PER CENT OF SURVIVORS OVER CONTROLS		P† VALUE
					MEAN	S.E.*		MEAN	S.E.*	
A	25	18 hr. before	5	245	47	22	> 0.1	12	5	< 0.05
B	25	5 hr. before	1	38	182			25		
C	25	30 min. before	12	515	164	38	< 0.001	55	6	< 0.001
D	15	30 min. before	5	207	139	25	< 0.01	20	5	< 0.01
E	5	30 min. before	5	207	20	21	> 0.8	4	2	> 0.2
F	25	5 min. before	4	198	155	25	< 0.02	26	11	< 0.01
G	25	1 min. before	2	95	46			0		
H	25	1 min. after	2	93	-52			-6		
I	25	5 min. after	3	137	-25			-4		
J	25	30 to 60 min. after	5	212	-2	23	> 0.9	-4	3	> 0.9
K	No injection		17	772	15	6		0	2	

*Standard error of the mean (= $\frac{\text{Standard deviation}}{\sqrt{n}}$).

†In each case comparison is made with Experiment K. P value was estimated by the method of Fisher¹¹ from the t value

$$t = \frac{\text{Difference of the means}}{\text{Standard deviation of the differences of the treated and Experiment K groups}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} :$$

n_1, n_2 = number of trials in the groups compared. P value of less than 0.05 indicates a significant difference in response to treatment.

injected at various time intervals prior to scalding. As shown in the table (Experiment A), ethanol injected eighteen hours before the burn resulted in an average increase in survival time of 47 per cent and an average decrease in mortality of 12 per cent. When compared with controls, the average increase in survival time was not within the range of significant activity, although an apparently significant number of mice survived longer than the control mice. On the other hand, ethanol administered between five and thirty minutes before the animals were scalded significantly increased survival time and reduced mortality (Experiments C and F). In a single experiment employing thirty-eight mice, a positive result was obtained with ethanol injected five hours before scalding (Experiment B). No activity was noted when ethanol was injected one minute before scalding, probably because there was insufficient time for absorption (Experiment G). Negative results were obtained with ethanol administered from one to sixty minutes after the burn (Experiments H, I, and J).

Effect of Ethanol on Bleeding Volume in Shock.—An appreciable reduction of the bleeding volume has been demonstrated in burn shock.^{10, 11} Since ethanol has prophylactic activity in shock, its influence on the bleeding volume after burns was investigated. As seen in Fig. 1, ethanol did not significantly influence the bleeding volume in unburned mice. Scalded mice given a control injection of 0.2 c.c. of isotonic salt solution showed a marked reduction of the bleeding volume. This reduction was prevented to a significant extent when ethanol was injected thirty minutes prior to scalding. The bleeding volume was unchanged when ethanol was given after the burn.

From these experiments, it is concluded that the prophylactic effect of ethanol is related to its ability to prevent the reduction of bleeding volume which occurs in mice shocked by scalding.

Effect of Ethanol on Local Fluid Loss.—As previously stated, the reduction in the effective circulatory volume in burn shock is believed to be due to two factors, local fluid loss at the site of the trauma and capillary atony.⁴ The

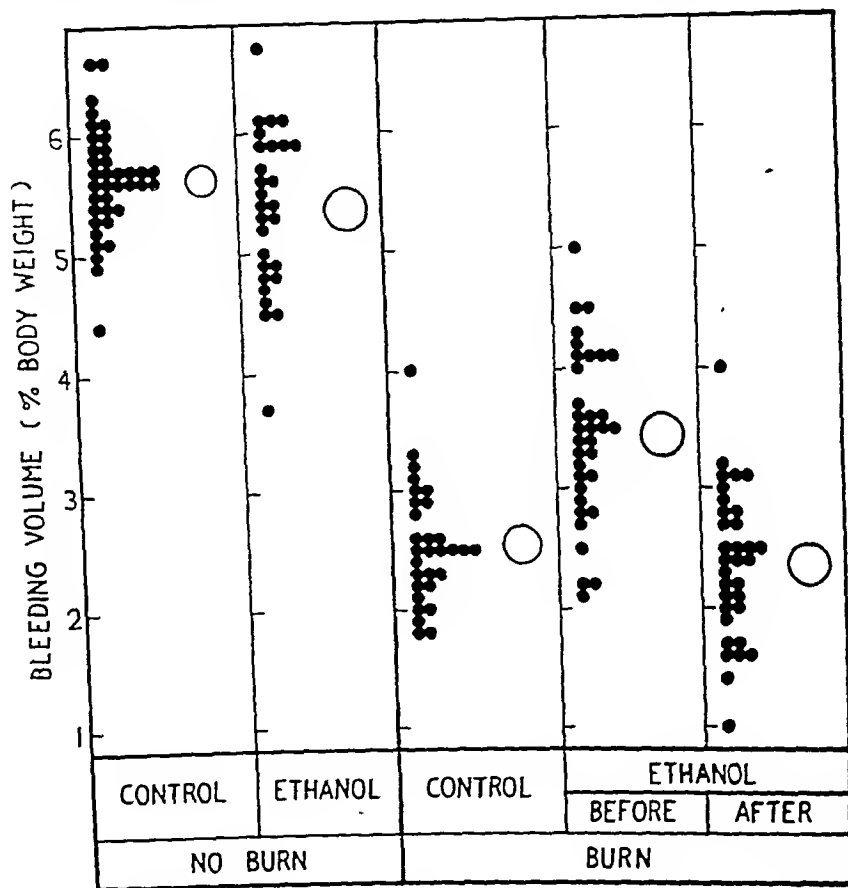


Fig. 1.—Effect of ethanol on bleeding volume in burn shock. Mice were scalded to the head at 65° C. for ten seconds. One group of burned animals was injected with 25 per cent ethanol thirty minutes before and a second group five minutes after, scalding. Unburned, control animals received isotonic salt solution and ethanol, respectively, and burned, control animals received only saline. All solutions were injected intraperitoneally in amounts of 0.2 c.c. Bleeding volumes in the unburned mice were taken from one to two hours after injection of the test solution and in the burned animals at a similar time interval after thermal injury. Each dot represents the response of a single mouse. The large circles represent the mean response; the diameter of each large circle equals three times the standard error of the mean.

antishock properties of ethanol would be expected to result from its ability to diminish local edema formation or capillary atony or both. The following experiment was performed to determine the effect of ethanol on local fluid loss (edema formation).

Fluid loss in the scalded parts was studied by a modification of Blalock's bisection method.⁸ One hindlimb of each of seventy-two anesthetized mice

was scalded at 65° C. for ten seconds. Approximately three hours after scalding, each animal was again anesthetized with ether, the bleeding volume determined, and the amount of fluid loss into the tissues ascertained by bisecting the animal, severing the hindquarters on each side, and comparing the weights of the two hindquarters. In observations on twelve unburned mice (Fig. 2), the average difference in weight of the hindquarters in terms of percentage of body weight was $0.39 \pm 0.07^*$ per cent, showing that the accuracy of the bisecting technique for mice equaled that for rats.⁸

Estimation of the amount of edema formation was made in two sets of animals, the one receiving 0.2 c.c. of 25 per cent ethanol intraperitoneally prior to scalding and the other receiving 0.2 c.c. of salt solution only. The results as shown in Fig. 2 were as follows: in forty-eight control burned mice, the average fluid shift to the scalded side was $5.02 \pm 0.15^*$ per cent; in twenty-four burned mice treated with ethanol, this value was $3.73 \pm 0.19^*$ per cent. The difference is statistically significant.

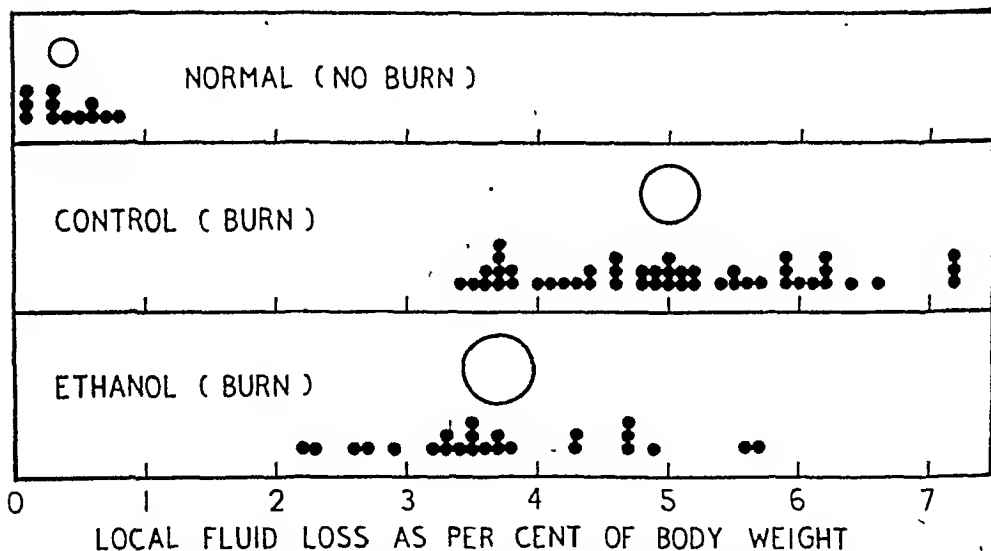


Fig. 2.—Effect of ethanol on local fluid loss. Mice were injected intraperitoneally with 0.2 c.c. of isotonic salt solution and 25 per cent ethanol, respectively, prior to burning a single hindlimb at 65° C. for ten seconds. Estimation of the amount of local fluid loss was made from two to four hours after the burn. Each dot represents the response of a single mouse. The large circles represent the mean response; the diameter of each large circle equals three times the standard error of the mean.

As shown in Fig. 2, the effect of preliminary treatment with ethanol was to reduce significantly the amount of edema in the scalded parts. From previous experience in this laboratory, when one hindlimb was scalded at 65° C. for ten seconds, death did not result within forty-eight hours in a single instance, in spite of the fact that the local fluid loss often amounted to as much as 5 per cent of the body weight.

Prophylactic Effect of Ethanol on Capillary Atony.—As described in previous publication, shock and death can be produced with little or no local fluid

*Standard error of the mean.

loss following a severe burn at $100^{\circ}\text{C}.$ ⁸ In this type of thermal trauma, it was demonstrated that the reduction in the circulatory blood volume was largely due to visceral capillary congestion which resulted from the operation of a toxic factor in the blood.⁴ The degree of capillary atony was ascertained by measuring the amount of blood retained in an organ, that is, liver, after exsanguination of the animal. Capillary atony also contributes to the production of shock in burns which are accompanied by considerable fluid loss. In mice scalded to the head at $65^{\circ}\text{C}.$ for ten seconds, the amount of hemoglobin retained in the liver was appreciably greater than in normal controls.⁴

In order to ascertain the effect of ethanol upon capillary atony, the following experiments were performed. Three groups of mice, each consisting of fifty-seven animals, were used; these groups were divided into six duplicate sets of nine or ten mice each. The first group consisted of animals which received an intraperitoneal injection of 0.2 c.c. of 25 per cent ethanol thirty minutes prior to scalding to the head at $65^{\circ}\text{C}.$ for ten seconds. The second group was scalded in a similar manner but received 0.2 c.c. isotonic salt solution instead of ethanol. The third group consisted of normal, unburned mice which served as controls to correct for the varying degrees of hemoconcentration noted after this type of burn. Bleeding volumes were determined by exsanguination; samples of blood were taken, and the livers were removed for estimation of hemoglobin. In the first two groups, this was done from three to four hours after the burn. The amount of hemoglobin retention in the exsanguinated livers was used as an index of the degree of capillary atony.

Method of Determining Hemoglobin in Liver: After testing published methods and experimenting with modifications, the following procedure was found to be the most satisfactory and expedient for making comparative measurements of the amount of hemoglobin retained in the tissue under investigation. Hemoglobin retained by the liver was extracted with a buffered salt solution and measured photometrically after conversion to cyanmethemoglobin.¹² The liver was finely minced with scissors and covered with a volume of buffered salt solution equal to ten times the weight of the tissue. This buffered salt solution was prepared by dissolving 300 Gm. of sodium chloride, 7.62 Gm. of sodium tetraborate (borax), 6.18 Gm. of boric acid, and 0.50 Gm. of saponin in distilled water, by diluting to 1 L., and by filtering. The pH of this solution varied between 7.05 and 7.15. Extraction with occasional shaking was continued for eighteen hours or more. The mixture was then filtered through Whatman filter paper No. 2. This procedure gave brilliant filtrates, so that there was no need for turbidity corrections. To the filtrate was added one drop of 20 per cent potassium ferricyanide to convert the hemoglobin into methemoglobin; the latter was converted to cyanmethemoglobin by the addition of one drop of a freshly prepared mixture of equal parts of 10 per cent sodium cyanide and 12 per cent acetic acid. The cyanmethemoglobin color was estimated with the Klett-Summerson photoelectric colorimeter, using Filter 54.

A few facts concerning the practical application of this and other methods are pertinent. Liver minced with scissors and leached for eighteen hours by the method described does not yield all of its color at the end of this period, since leaching of the residue left by filtration for an additional eighteen hours yields an added 10 per cent of cyanmethemoglobin color. Nevertheless, leaching for an eighteen-hour period does provide satisfactory and uniform values when comparative observations are made. Tests performed in this manner, with like amounts of tissue taken from the same organ, gave hemoglobin values within 5 per cent of each other. This method was found suitable for making comparative

estimations of hemoglobin in blood and in tissue other than liver. The use of the Waring blender for mincing the liver was found to be unsatisfactory; low recovery values for hemoglobin were obtained, because most of the pigment was absorbed by the colloidal mass. Methods designed to recover hemoglobin as iron by wet ashing the tissue were tedious and difficult to evaluate due to the presence of variable amounts of nonhemoglobin iron.

As shown in Table II, 91 per cent more hemoglobin was retained in the liver of the saline-treated scalded mice than in the unburned controls and only 40 per cent more in the ethanol-treated animals. Statistical analysis shows that the difference between these figures is significant. Thus, the administration of ethanol significantly reduced the amount of hemoglobin usually retained in the liver after scalding.

TABLE II. PROPHYLACTIC EFFECT OF ETHANOL ON HEMOGLOBIN RETENTION IN BURN SHOCK

EXPERIMENT	NUMBER OF LIVERS COMPARED IN EACH GROUP	HEMOGLOBIN RETENTION AS PER CENT OF VALUE IN UNBURNED, EXSANGUINATED GROUP	
		ETHANOL (BURNED)	SALINE (BURNED)
1	10	137	218
2	9	116	204
3	10	145	216
4	9	145	143
5	9	125	141
6	10	174	216
Mean \pm S.E.*		140 \pm 8	191 \pm 15
P value*		< 0.05	

*Symbols and method of calculation same as Table I.

These observations confirm previous ones, that scalding leads to visceral congestion (increase in hemoglobin retention) in the liver, and lead to the conclusion that ethanol administered in the manner described owes its antishock action in part to a reduction in visceral capillary atony.

DISCUSSION .

By employing a standard procedure for producing burn shock in mice, which consists of scalding the anesthetized animals to the head at 65° C. for ten seconds, it was shown that the intraperitoneal injection of ethanol prior to scalding increased the average survival time and diminished the average mortality rate. The results with ethanol parallel those previously obtained with ethanol-free 15-unit liver extract. Ethanol, like liver extract, had no antishock activity when administered after the thermal injury.

In experiments reported in this and in previous communications, it was shown that when mice were scalded in the manner described the bleeding volume in the shocked animals was significantly reduced. A decreased bleeding volume has been accepted as an index of reduced circulating blood volume, and it is generally agreed that the reduction in the circulatory volume is largely responsible for the chain of events which leads to shock in burns. It was shown that ethanol, when injected in suitable concentration *prior* to scalding, curtailed the usual reduction of bleeding volume, and this fact explains the increase in survival time and diminution in mortality. On the other hand, the reduction in bleeding volume in scalded mice was not influenced

when ethanol was given after the burn, a fact which explains the failure of ethanol to protect the animals when this substance was administered after the trauma.

Two factors have been found to be responsible for the reduced circulating volume in burn shock; the one, fluid loss at the site of trauma and the other, capillary atony, due to a toxic agent.⁴ The influence of ethanol on both of these factors was investigated. By comparing the amount of local fluid loss in burned mice given ethanol with that in control, burned animals, it was shown that ethanol curtailed the amount of fluid lost in a burned limb when this substance was injected prior to scalding but not when administered after the injury.

The mechanism whereby ethanol inhibits local fluid loss or edema formation requires further investigation. In a companion paper, data are presented which show that barbiturates, as well as other substances, have antishock properties paralleling those of ethanol and that the prophylactic effects are due in part to the curtailment of edema formation. A discussion of the factors which may be responsible for the observed inhibitory effects upon local fluid loss will be found in that paper.¹³

There remains for discussion, in considering the mechanism of the antishock action of ethanol, the effect of this substance upon the operation of a toxic factor. In previous experiments performed in this laboratory,⁴ it was shown that the pooling of large amounts of blood in atonic visceral capillaries contributes to the fall in the circulatory volume and that this congestion is due to a circulating toxic factor.

The degree of visceral congestion was estimated by comparing the amount of blood retained in the liver of burned, exsanguinated mice with that in unburned controls. In scalded mice there was a notable retention of blood in the hepatic capillaries. Experiments showed that the prophylactic administration of ethanol resulted in a significant reduction of the amount of blood retained in the liver of scalded animals but did not influence the amount of blood retained in the liver of unburned controls. The manner in which ethanol counteracts capillary atony is not known. It is possible that this substance neutralizes the hypothetic humoral toxic factor which causes capillary atony, or it may decrease the responsiveness of the capillaries to this factor.

SUMMARY AND CONCLUSIONS

Intraperitoneal injection of ethanol increases survival time and diminishes mortality in mice subjected to a standardized thermal injury. This agent has a prophylactic effect when administered before the burn but is without antishock action when given after the trauma.

In burn shock, the circulating blood volume as represented by the bleeding volume is significantly reduced, and the prophylactic administration of ethanol curtails the expected reduction. Ethanol counteracts shock (1) by diminishing local fluid loss (edema formation) and (2) by reducing visceral congestion due to capillary atony.

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THE ANTISHOCK ACTION OF CERTAIN DRUGS IN BURNED MICE

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IN A companion paper,¹ we reported that ethanol increased survival time and diminished mortality in mice subjected to a standardized shock-producing scalding procedure. In order to be effective as an antishock agent, it was necessary to administer ethanol before the burn, for it had no therapeutic or prophylactic action when given after the trauma.

In previous studies, it was shown that two major factors contributed to the initiation of scald shock; the one, fluid loss at the site of thermal injury and the other, visceral congestion due to capillary atony.² Each of these factors in suitable degree, separately or in combination, leads to a reduction in the circulating blood volume.

In order to investigate the mechanism whereby ethanol exerts its antishock action, studies were made to ascertain the effect in burned animals of prophylactic administration of this agent upon the circulatory volume, the amount of local fluid loss, and the degree of visceral congestion. From these experiments, it was concluded that ethanol counteracts shock by curtailing local fluid loss and by reducing visceral congestion.

In a previous report, it was shown that ethanol-free 15-unit liver extract displays the same kind of antishock action as was subsequently demonstrated for ethanol.³ The purpose of the present communication is to report investigations upon compounds related either chemically or pharmacologically to ethanol. In these studies, similar antishock activity was demonstrated for sodium pentobarbital, morphine, histamine, acetone, glycerol, and propylene glycol. These substances were selected for the following reasons: glycerol and propylene glycol because they are alcohols; acetone because of the similarity of its pharmacologic action to that of ethanol; sodium pentobarbital and morphine because of their narcotic properties; and histamine because of its vasodilator activity. The mechanism of their action was investigated by the methods described in the companion paper on ethanol.¹

EXPERIMENTS AND RESULTS

Effect of Various Drugs on Survival Time and Mortality in Scald Shock.—

Glycerol and Propylene Glycol: In six trials, in which glycerol (25 per cent) was injected intraperitoneally into mice thirty minutes prior to scalding to the head at 65° C. for ten seconds, there was a significant increase in survival time and in the number of survivors during the forty-eight hour period

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TABLE I. EFFECT OF VARIOUS DRUGS ON SURVIVAL TIME AND MORTALITY IN BURN SHOCK

EXPERIMENT	DRUG	AMOUNT INJECTED BEFORE BURN	NUMBER OF TRIALS	TOTAL NUMBER OF MICE	PER CENT INCREASE IN AVERAGE SURVIVAL TIME OVER CONTROLS		P† VALUE	INCREASE IN PER CENT SURVIVORS OVER CONTROLS		P† VALUE
					MEAN	S.E.*		MEAN	S.E.*	
A	Glycerol	0.2 c.c. of 25%	6	252	123	43	< 0.02	11	6	< 0.05
B	Propylene glycol	0.2 c.c. of 25%	6	255	92	28	< 0.02	12	5	< 0.05
C	Acetone	0.2 c.c. of 25%	5	212	169	55	< 0.01	23	7	< 0.01
D	Sodium pentobarbital	1 mg. in 0.2 c.c.	7	280	255	70	< 0.01	20	7	< 0.01
E	Morphine sulfate	0.5 mg. in 0.2 c.c.	7	280	371	41	< 0.01	30	7	< 0.01
F	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	7	280	326	53	< 0.01	20	2	< 0.01
G	No injection		17	772	15	6		0	2	

*Standard error of the mean ($= \frac{\text{Standard deviation}}{\sqrt{n}}$).

†In each case comparison is made with Experiment G. P value was estimated by the method of Fisher's from the t value.

($t = \frac{\text{Difference of the means}}{\text{Standard deviation of the differences of the treated and Experiment G groups}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$);

n_1, n_2 = number of trials in the groups compared. P value of less than 0.05 indicates a significant difference in response to treatment.

of observation (Table I, Experiment A). Similar results were obtained with propylene glycol (25 per cent), as shown in Table I, Experiment B.

Acetone: In five trials, with a 25 per cent solution, it was found that acetone was as effective as ethanol in diminishing mortality and increasing survival time (Table I, Experiment C).

Sodium Pentobarbital: In seven trials, with the prophylactic injection of 1 mg. in 0.2 c.c. of physiologic salt solution, there was a significant increase in survival time and in the number of survivors (Table I, Experiment D). Sodium pentobarbital in the dosage used exhibited a greater narcotic effect than 0.2 c.c. of a 25 per cent solution of ethanol.

Morphine: Morphine sulfate (0.5 mg. in 0.2 c.c. of physiologic salt solution) was injected intraperitoneally from thirty to forty minutes prior to scalding. In seven trials, the results showed a significant reduction in mortality and an increase in survival time (Table I, Experiment E). The dose of morphine administered was sufficient to maintain the curved-tail response⁴ for the period prior to scalding.

Histamine: Seven trials were performed in which histamine diphosphate (0.1 mg. as histamine base in 0.2 c.c. of physiologic saline) was injected intraperitoneally into each mouse from five to eight minutes prior to scalding. This short interval was chosen because the vasodilator action of histamine is comparatively brief. The results showed that histamine increased survival time and decreased mortality to a significant degree (Table I, Experiment F).

Effect of Various Drugs on Local Fluid Loss and Bleeding Volume in Scald Shock.—A major factor which contributes to the reduction in the circulatory blood volume in burn shock is fluid loss or edema at the site of the trauma. Ethanol administered prior to scalding was found to curtail the usual reduction in the circulating volume and to diminish the amount of local edema formation.¹

Effect on Local Fluid Loss and Bleeding Volume of Drugs Administered Before Burning: In order to investigate the effect of morphine, sodium pentobarbital, and histamine upon local fluid loss and the circulatory volume in burn shock, the following experiments were performed. One hind limb of each of 120 anesthetized mice was scalded at 65° C. for ten seconds. Approximately three hours after scalding, each animal again was anesthetized with ether, the bleeding volume determined, and the amount of fluid loss into the tissue ascertained by the method previously described.² Estimation of the amount of local edema formation was made on four groups of burned animals, all of which had been given a test substance intraperitoneally thirty minutes prior to scalding. The first group was given morphine sulfate; the second, sodium pentobarbital; the third, histamine; and the fourth, a control injection of physiologic salt solution.

As shown in Table II, the effect of prophylactic treatment with morphine, sodium pentobarbital, and histamine was to reduce significantly the amount of edema formation in the burned limb. The expected diminution of the bleeding volume was prevented to a significant extent. It should be noted, however, that in this experiment in which only one limb was burned the reduction in bleeding volume induced by the thermal trauma was much less than in animals scalded to the head.³

TABLE II. EFFECT ON LOCAL FLUID LOSS AND BLEEDING VOLUME OF DRUGS ADMINISTERED THIRTY MINUTES BEFORE THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)				BLEEDING VOLUME (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P†	NUMBER OF MICE	MEAN	S.E.*	P†
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	24	3.52	0.19	< 0.01	24	3.79	0.12	< 0.01
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	24	3.99	0.21	< 0.01	24	3.90	0.10	< 0.01
C	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	24	3.71	0.17	< 0.01	24	3.63	0.14	< 0.05
D	Saline	0.2 c.c.	48	5.02	0.15	< 0.01	48	3.48	0.07	< 0.01
E	No burn		12	0.39	0.07	< 0.01	37	5.63	0.07	< 0.01

*Symbols and method of calculation same as Table I.
†In each case comparison is made with Experiment D.

TABLE III. EFFECT ON LOCAL FLUID LOSS OF DRUGS ADMINISTERED FROM FIVE TO EIGHT MINUTES AFTER THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P† VALUE
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	24	4.07	0.17	> 0.4
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	24	3.90	0.17	> 0.1
C	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	24	3.79	0.18	> 0.05
D	Saline	0.2 c.c.	24	4.24	0.19	

*Symbols and method of calculation same as Table I.

†In each case comparison is made with Experiment D.

TABLE IV. EFFECT ON ABSORPTION OF EPIDERMAL FLUID OF DRUGS ADMINISTERED SIXTEEN HOURS AFTER THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P† VALUE
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	15	3.29	0.22	> 0.7
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	15	2.98	0.18	> 0.1
C	Saline	0.2 c.c.	15	3.23	0.21	

*Symbols and method of calculation same as Table I.

†In each case comparison is made with Experiment C.

Effect on Local Fluid Loss of Drugs Administered Immediately After Burning: The same drugs were used as in the previous experiment (Table II, Experiment A), but they were given from five to eight minutes after scalding a single hind limb at 65° C. for ten seconds. The mice were biseected approximately four hours after trauma. As shown in Table III, each of these compounds failed to inhibit local edema formation.

Effect of Drugs on Absorption of Edema Fluid: As shown in the two previous experiments (Table II, Experiments A and B), sodium pentobarbital and morphine sulfate curtailed local edema formation when they were administered before, but not after, the burn. The following experiment was performed to

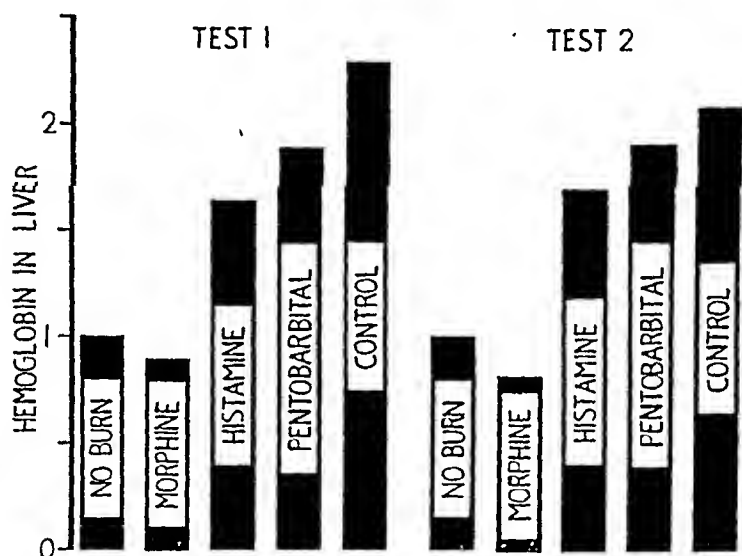


Fig. 1.—Effect of various drugs on hemoglobin retained in the liver. Morphine sulfate (0.5 mg. in 0.2 c.c. saline), histamine diphosphate (0.1 mg. base in 0.2 c.c. saline), sodium pentobarbital (1 mg. in 0.2 c.c. saline), and 0.2 c.c. saline (control) respectively, were injected intraperitoneally into mice (six mice per group). Thirty minutes after injection, both hind limbs of each mouse were scalded at 100° C. for twenty seconds, and the animals were exsanguinated three hours later. The amount of hemoglobin retained in the liver of each group of burned mice was compared with that in a parallel group of unburned animals.

ascertain whether or not either of these drugs would influence the absorption of fluid once edema had developed in the traumatized limb. One hind limb of three groups of fifteen mice each was burned at 65° C. for ten seconds. After sixteen hours, mice in two of the groups received an intraperitoneal injection of sodium pentobarbital and morphine sulfate, respectively, and those in the third group physiologic salt solution only. The dosage of each of the drugs was the same as in the previous experiments. From three to five hours after injection, the animals were sacrificed, and the amount of edema was determined by the bisection technique. As shown in Table IV, neither sodium pentobarbital nor morphine sulfate had any effect on the resorption of edema fluid.

Effect of Drugs on Capillary Atony.—In addition to local fluid loss, a second factor, that of capillary atony, is believed to contribute to the initiation of

burn shock.² This factor operates by causing stagnation of blood in atonic visceral capillaries, with a consequent reduction of the circulating blood volume.

In order to study the effect of sodium pentobarbital, morphine, and histamine upon capillary atony, the following experiments were performed. In each of two tests, five groups of six mice each were used. In four of the groups in each test, both hind legs of each animal were scalded at 100° C. for twenty seconds. Sodium pentobarbital, morphine sulfate, histamine, and physiologic salt solution, respectively, were injected intraperitoneally thirty minutes prior to the burn. The animals in the fifth group in each test were not subjected to a burn. The mice were exsanguinated at the end of three hours and the livers removed for estimation of hemoglobin. The amount of hemoglobin retention in the exsanguinated livers was used as an index of the degree of visceral congestion due to capillary atony. The method of estimating hemoglobin in the liver was described in the companion report.¹

As shown in Fig. 1, morphine reduced the amount of hemoglobin retained in the liver of burned mice. The effect of pentobarbital and histamine upon the hemoglobin retention was not significant.

DISCUSSION

After it was shown that ethanol, prophylactically administered, increased survival time and diminished mortality in mice subjected to a standardized thermal injury,¹ other compounds related either chemically or pharmacologically were investigated for antishock action. Morphine and sodium pentobarbital (drugs having narcotic properties), histamine (a vasodilator), acetone (related chemically to ethanol), glycerol, and propylene glycol (alcohols) were all found to increase survival time and the number of survivors.

The mechanism of the antishock action of morphine, sodium pentobarbital, and histamine was investigated by observing their effect upon two major factors which are implicated in the initiation of burn shock, namely, local fluid loss and visceral congestion due to capillary atony.

The effect of these compounds upon edema formation in a burned limb was investigated. Each of these drugs curtailed the amount of fluid loss in the burned extremity, but only if administered before the thermal injury. None of these drugs had any effect upon the development of edema when injected immediately after the burn, nor did these agents hasten absorption of edema fluid when given after swelling of the scalded limb was well established.

Animals in which a single hind limb was burned exhibited a small, but significant, reduction in the circulatory blood volume as represented by the bleeding volume, and each of these drugs curtailed to some extent the expected reduction due to thermal injury.

A number of observers have noted antishock activity for barbiturates in animals subjected to manipulation of the intestines, to burning of the intestines, or to scalding of the skin.⁶⁻¹⁰ Although Beecher and McCarrell⁶ report sodium pentobarbital to be effective when injected after a burn in rabbits, we found this barbiturate, as well as morphine, histamine, and ethanol active only if given prior to the trauma. Beecher and co-workers^{7, 8} attributed the thera-

peutic effect of the barbiturate to a curtailment of fluid loss from the burned surface. To explain this result, these authors suggested two possibilities; the one, reduction by the barbiturate of the flow of capillary filtrate due to capillary contraction and the other, lowering of the capillary pressure in the burned tissue. It is unlikely that the first of these possibilities holds for histamine or ethanol, inasmuch as these substances are known to dilate capillaries. Before the second explanation can be accepted or disproved, direct measurements of capillary pressure would be required. Another possibility that suggests itself is that barbiturates and the other drugs studied may have a specific action in decreasing capillary permeability at the site of injury. There is no clear-cut evidence for this, although Polderman and Beecher¹¹ have observed that the flow of cervical lymph is less under barbiturates than under ether. In summary the mechanism whereby these drugs prevent local fluid loss has not been satisfactorily explained.

The influence of each of these three substances upon visceral congestion due to capillary atony was investigated by estimating the amount of blood (hemoglobin) retained in the liver of exsanguinated mice. In burned animals which had received a prophylactic injection of morphine, less hemoglobin was retained in the liver than in untreated, burned controls. Sodium pentobarbital and histamine were without significant effect. Previously, Castleman, who examined the tissue in the experiments performed by Beecher and McCarrell,⁸ had noted that barbiturate diminished the amount of capillary congestion in the duodenal mucosa of rabbits with experimental skin burns.

It has been suggested by some investigators that histamine or related substances may be implicated in the production of the shock state.¹²⁻¹⁴ In the present study, histamine was injected in amounts capable of producing some of the circulatory phenomena of shock;¹⁵ yet an inhibitory action was exerted upon the expected reduction of the circulatory blood volume and the rate of local edema formation in burns. This would indicate that, in so far as burn shock is concerned, histamine is probably not the toxic factor involved in the production of the shock syndrome.

From the experiments which form the subject of this communication, it was concluded that morphine, sodium pentobarbital, and histamine owe their antishock action in burns, at least in part, to their inhibitory effect upon edema formation at the site of the burn, and that morphine in addition counteracts visceral congestion due to capillary atony.

SUMMARY AND CONCLUSIONS

Sodium pentobarbital, morphine, histamine, acetone, glycerol, and propylene glycol display antishock activity similar to that of ethanol. These substances increase survival time and diminish mortality in mice subjected to a standardized thermal injury.

The mechanism whereby morphine, pentobarbital, and histamine counteract shock was investigated by observing the effect upon two major factors implicated in burn shock, namely, local fluid loss and capillary atony.

Each of these drugs curtails edema formation at the site of the burn, but only if injected before the trauma. They neither prevent the development of edema nor accelerate the absorption of fluid when given after the burn. Morphine in addition counteracts visceral congestion due to capillary atony.

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THERAPEUTIC INDUCTION OF FEVER AND LEUCOCYTOSIS USING A PURIFIED TYPHOID PYROGEN

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A PURIFIED somatic antigen isolated from cultures of *Eberthella typhosa* grown on a synthetic medium was found to produce, in addition to the immunologic response, a febrile reaction followed by a secondary leucocytosis when injected intravenously in rabbits^{1, 2} and man.³ These properties of the antigen suggested its clinical application as a pyrogen in the induction of fever for therapeutic purposes in place of the whole typhoid-paratyphoid vaccine now generally employed.

MATERIALS AND METHODS

The original preparation of purified somatic typhoid antigen² had been stored in the refrigerator at 4° C. for from one to five years before use in these experiments. It consisted of a distilled water suspension containing 5 mg. of the antigen per cubic centimeter. The antigen, diluted in saline, was administered intravenously into the median basilic vein. Rectal temperature determinations were made at hourly intervals, and leucocyte counts were made at frequent intervals during the febrile paroxysms. Thirteen individuals, eight men and five women, eleven of whom had neurosyphilis, were selected for the induction of therapeutic fever.

RESULTS

A summary of the results obtained in six patients representative of the series is presented in Table I. The temperature and leucocytic response of a typical paroxysm are reproduced in Fig. 1. Following an adequate dose of the pyrogen, the temperature began to rise within from one-half to one hour after the injection and reached its fastigium in two or three hours, falling again to normal usually within from seven to eight hours. In Patients 2 and 4, the initial dose was small, since the toxicity of the preparation for man was not known. As a safety precaution the increments in the amount of antigen added in each succeeding dose were small. In subsequent cases an initial dose of 0.001 mg. was found to be safe. At first, the dose was usually doubled on each consecutive injection. It was soon found that this amount was not sufficient to obtain a consistently adequate febrile response. When a three- to fivefold increment in dosage was used, the results were usually satisfactory, as demonstrated with Patients 5, 7, 12, and 13.

With an adequate dose, the rectal temperature remained elevated at 103° F. or higher for from two to twelve hours, depending on the amount of pyrogen

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TABLE I. FEBRILE AND LEUCOCYTIC RESPONSE FOLLOWING INJECTION OF TYPHOID PYROGEN

PATIENT	DAYS AFTER INJEC- TION	AMOUNT OF PYROGEN (MG.)	MAXIMUM TEMPERATURE RECTALLY (° F.)	FEVER OF 103° F. OR MORE	LEUCOCYTE COUNT	COUNTED AFTER INJEC- TION (HOURS)
2	0	0.0001	100.1	0	—	—
	1	0.0002	101.8	0	9,000	3
	2	0.0003	101.2	0	—	—
	3	0.001	101.2	0	11,000	5
	6	0.002	101.6	0	—	—
	7	0.004	101.8	0	—	—
	8	0.01	104.4	0	7,000	5
	10	0.01	102.4	3	11,000	6
	11	0.01	102.0	0	8,000	7
	13	0.01	102.4	0	11,000	10
	14	0.01	101.2	0	8,500	5
	15	0.05	102.2	0	9,500	9
	16	0.10	102.6	0	9,500	5
	17	0.20	104.8	5	10,000	7
4	22	0.20	104.0	2	17,000	10
	23	0.20	103.6	2	14,500	8
	0	0.0001	101.6	0	10,000	5.5
	1	0.0002	102.0	0	9,000	5.5
	2	0.0005	101.6	0	9,000	5.5
	3	0.0025	104.6	4	—	—
	5	0.01	104.6	4	7,000	5.5
	7	0.05	105.0	5	—	—
	9	0.04	103.4	3	11,000	6
	11	0.1	105.2	5	28,000	9.5
	13	0.1	103.4	2.5	17,000	5.5
	15	0.3	105.6	5	11,000	5
	17	0.30	104.6	6	11,000	5
5	0	0.0002	100.2	0	9,500	6
	2	0.001	103.0	1	10,000	5
	4	0.007	105.0	2.5	22,000	13.5
	6	0.01	103.4	2.5	15,000	13.0
	9	0.05	102.6	0	10,500	6.0
7	0	0.0002	99.4	0	10,000	10.5
	1	0.002	101.0	0	17,000	5
	2	0.02	105.0	4	22,000	11
	5	0.02	103.0	2	29,000	12
	7	0.1	102.4	0	19,500	10
	13	0.5	105.0	3	37,500	14
	15	0.5	101.6	0	35,500	10
12	0	0.001	103.0	1	—	—
	1	0.005	103.8	3	—	—
	2	0.01	103.0	1	—	—
	3	0.05	105.0	6	—	—
	5	0.15	104.8	5	—	—
	7	0.6	105.0	8.5	—	—
	9	1.8	104.0	8.5	—	—
	12	7.2	105.4	9	42,500	9
	14	5.7	105.4	8	33,700	9
13	0	0.0005	99.8	0	—	—
	1	0.002	105.2	5	—	—
	2	0.005	103.8	3.5	—	—
	3	0.025	104.6	6	—	—
	5	0.1	105.8	4	—	—
	7	0.3	105.0	6	—	—
	9	0.9	104.6	5.5	31,800	9
	12	3.6	105.8	7	29,000	6
	14	14.4	105.4	12	—	—

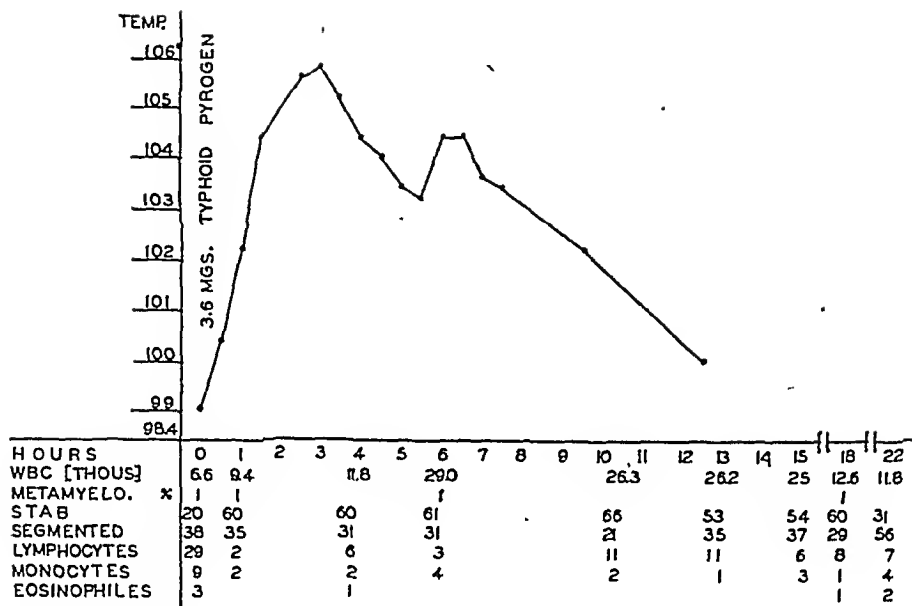


Fig. 1 (Patient 13).—Typical febrile and leucocytic response following injection of typhoid pyrogen.

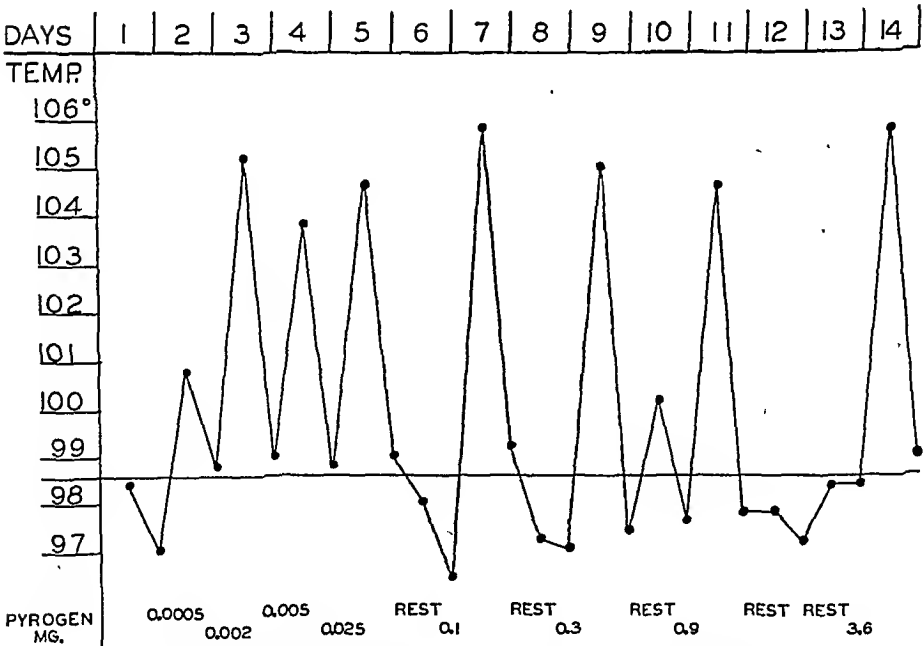


Fig. 2 (Patient 13).—Fever curve obtained with typhoid pyrogen.

given and its relation to the dosage employed in the previous injection. When a fivefold or greater increase in dosage was used, the temperature rose to 104° F. or higher in most instances. However, as the amount of pyrogen given in a single dose grew larger (Patients 12 and 13), a temperature of 104° F. or more could be obtained by only tripling or quadrupling the preceding dose. These data show that the patients developed a tolerance for the fever-producing action of the substance.

Fig. 2 represents a typical course of febrile paroxysms produced in Patient 13. The lowest and the highest temperature of each paroxysm are recorded.

The leucocytosis developed within from three to five hours after the injection of the pyrogen and continued to increase for as long as fourteen hours. The total count often reached values of from 20,000 to 30,000 per cubic millimeter and occasionally higher, as shown in Fig. 1 and Table I. The differential counts showed that this elevation was due to an increase in the granulocytes, a large number being stab forms. The total leucocyte count often remained elevated for twenty-four hours or longer, particularly when larger doses of the pyrogen were used.

The systemic symptoms accompanying the administration of an effective dose of the antigen were quite uniform. Within from thirty to forty minutes after the injection, the patient began to feel chilly and developed a rigor of varying intensity which lasted for from twenty-five to thirty-five minutes. This was followed by generalized aching sensations which at times were accompanied by localized pain in the head, legs, back, and other parts of the body. The patient perspired during the height of the fever. Nausea and occasional vomiting also occurred. With the subsidence of the symptoms, there followed a feeling of fatigue and exhaustion. Only slight to moderate variations³ in the blood values of glucose, chlorides, and carbon dioxide combining power appeared during the paroxysms, indicating that dehydration was not a factor in the development of the fever and leucocytosis. The ingestion of fluids during febrile periods probably aided in keeping the variations at a minimum.

DISCUSSION

A purified pyrogenic antigen isolated from cultures of *E. typhosa* grown in a synthetic medium has been shown to be an efficient, reliable substance for the induction of therapeutic fever. When an effective dosage has been established by preliminary testing, a series of febrile reactions of desired intensity can be induced with a single injection by increasing each consecutive dose by three- to fivefold as indicated in the results obtained in Patients 12 and 13.

The stability of the pyrogenic antigen makes it possible to use the standardized material for long periods of time. Patients 12 and 13 were treated with the same lot of pyrogen five years after Patients 2, 4, 5, and 7, with comparable results. During the five-year interval, the pyrogenic material was usually stored in the refrigerator at 4° C. but was kept at room temperature for as long as four days on several occasions. These results suggest that a large stock of the pyrogenic antigen can be prepared, standardized, and stored for long periods of time as needed for use in the induction of fever.

The stability and uniformity of the pyrogen and its effectiveness in small single doses make it preferable to the whole bacterial typhoid-paratyphoid vaccine now employed for the same purpose, which varies in its febrile inducing properties and which often requires multiple injections to obtain a single satisfactory fever response.⁴ In addition, different lots of the bacterial vaccine have variable potencies in the induction of fever.

The marked leucocytosis which follows the injection of the pyrogen is probably a valuable adjunct in the therapeutic use of the substance.

SUMMARY

1. A pyrogenic somatic antigen has been isolated from cultures of *E. typhosa* which induces a febrile response in man when administered intravenously. A fever of from 103 to 105° F. follows doses of from 0.001 to 0.002 mg. To obtain similar results with subsequent injections, the amount of the pyrogen must be increased by three- to fivefold.

2. A granulocytic leucocytosis, with large numbers of nonfilament type of cells, accompanies and follows the febrile response.

3. When a given preparation of the pyrogen is standardized and stored in the refrigerator, it retains its potency for at least five years.

The authors wish to thank Dr. J. Gambesie and Dr. M. S. Ostrum, for their valuable assistance.

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COLORIMETRIC DIAGNOSTIC TEST FOR MALARIA

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INTRODUCTION

IN CHRONIC malaria and malaria in which parasites are not observed in stained blood films; it is important to have another means to aid in the diagnosis of the disease.

In 1928 Henry¹ described serologic tests based on the observation that sera from patients with malaria flocculate metharsenate of iron and melanotic pigment. The reaction containing iron was designated ferroflocculation and that which contained melanin and proved to be more sensitive, the melanoflocculation reaction. Grieg, van Rooyen, and Hendry² later made a complete study of the test. These investigators found the following disadvantages in the use of melanin prepared from ox choroid: (1) reactions from other protein substances, (2) inability to achieve complete uniformity in preparation of different batches of antigen, and (3) precipitation of stock choroid extract on storage. They prepared a melanin solution by hydrolysis of human hair, and the final extraction gave a solution which contained a fixed amount of pigment and remained stable. With this melanin extract they were able to change the technique of the test by discontinuing the preliminary titrations, increasing the sensitivity of the test, and using direct readings rather than electrometric devices. Even with this improvement the test was never used extensively, due to many false positive reactions.

Pewny,³ in 1918, and Zieman,⁴ in 1924, first attempted to demonstrate a precipitin reaction in malarial infections with limited success. In 1927 Taliaferro and co-workers⁵ reported success with this type of reaction by using an antigen prepared from human parasites obtained from placentas. However, in a second report⁶ they were not able to confirm their former findings fully. Row⁷ later improved the test by preparing the antigen from malarial culture tubes of *Plasmodium vivax*. He also simplified the technique to obtain a strong uniform constant precipitin reaction in his hands. The difficulty in the preparation of sufficient quantities of antigen and the tendency of the test to give false negative reactions made this method impractical.

Coggeshall and Eaton⁸ reported another diagnostic aid, the complement fixation reaction for chronic and apparently submicroscopic malaria. Their original work was done with monkey malaria, but later they were able to show similar results on human malaria⁹ using the same antigen prepared from the monkey parasite *Plasmodium knowlesi*. Although specific in nature, the test is

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not refined sufficiently to detect all known positive cases. *Plasmodium gallinaceum* appears to be a fairly successful parasite for antigen in complement fixation but does not produce as sensitive an antigen as *P. knowlesi*, either extracted or used whole. Group studies in this laboratory, using *P. gallinaceum* antigen on known patients with malaria, gave approximately 35 per cent positive reactions. As yet the human malarial parasite has not been cultured,⁹ so that sufficient quantities are available for a specific antigen.

All serologic reactions are basically the combination between antigen and antibody. The antigen in each case is usually specific, exceptions being those in the precipitation and complement fixation techniques in the detection of syphilis, in which some extract of beef heart is used, sheep cells in the heterophile reaction for infectious mononucleosis, etc. These serologic reactions are based primarily upon the coagulation and precipitation of a lyophobic colloidal dispersion in the presence of the serum containing specific antibodies or in some instances in the presence of a substance enhancing the differentiation of positive and negative sera.

Any type of test, either serologic or chemical, should be sufficiently sensitive to detect the weak positive or slight changes in the sera and should be specific for the disease without producing false positives in sera of patients with other diseases or normal individuals. The afore-mentioned tests do not meet these requirements, and it is apparent that an improved serologic or chemical test should be found which would enable one to follow the disease throughout its course.

The object of the present study was to develop a test, serologic or chemical, which would aid in the diagnosis of malaria. Much preliminary work was done in purifying *P. gallinaceum* antigen in attempts to improve the sensitivity and specificity, but it was found that purification did not increase the number of positive reactions in known positive sera, whether they were used in precipitation or complement fixation techniques. Melanin, used in previously mentioned tests,¹ presents in colloidal solution properties similar to those of solutions of certain colloidal dyes. Since colloidal dyes have been shown to combine quantitatively with proteins^{10, 11} under certain conditions, and since they also provide a convenient basis for a colorimetric evaluation of the altered dye solutions, it was felt that the modified characteristics of malaria serum occurring during the acute and chronic phases of the disease might be demonstrated by the use of such dyes.

GENERAL METHODS

In the earliest tests using dyes (Evans blue T1824, atabrine, methylene blue, neutral red, brilliant cresyl blue, and Congo red), concentrations of from 0.1 to 1.0 per cent were tried in varying amounts with known positive and negative malarial sera. Although promising, no consistent results were obtained in these preliminary titrations. It was thought that electrolytes might enhance the activity of the dye, and further titrations were carried out using these substances. Varying concentrations of NaCl were tried, and in some of the titrations it was possible to increase the activity of the dye; however, the action did not appear to be constant.

Of the several dyes (indicators) and electrolytes* (enhancers) used, Congo red, a colloidal dye, and quinine HCl† gave the most constant and consistent results in the presence of malarial and nonmalarial sera. The following report contains data based on the use of a colorimetric test involving Congo red, quinine HCl, and malarial serum.

APPARATUS

The following equipment and chemically clean glassware are suggested for the most effective performance of the test. Proper and careful dilution is also essential for obtaining the best results.

Test Tubes.—

1. 13 by 100 mm.
2. 25 by 100 mm.
3. Graduated cylinders, 100 c.c.
4. Pipettes
 - 1 c.c. to 0.01 c.c. graduation
 - 10 c.c. to 0.1 c.c. graduation
5. Spectrophotometer‡
6. Cuvettes, 19 by 105 mm.

Chemicals.—

1. Congo red§
2. Quinine HCl, 0.3 per cent aqueous sol

Sera.—Cell-free, nonhemolyzed, nonchylous, and noninactivated.

PROCEDURE

One cubic centimeter of a 0.1 per cent solution of aqueous NaR (Congo red) is placed in a test tube (13 by 100 mm.), and 0.5 c.c. of the noninactivated serum to be tested is added. This mixture is vigorously agitated. Two cubic centimeters of quinine hydrochloride are then added to the Congo red serum mixture, and complete mixing is obtained by inversion of the test tubes. The rack containing the tubes is allowed to remain at room temperature for forty-five minutes. The tubes are centrifuged for ten minutes at from 3,500 to 4,000 r.p.m. One cubic centimeter of the clear supernatant is transferred to a graduated cylinder and diluted to 60 c.c. with distilled water. One drop of N/10 NaOH is added to the diluted mixture. A 19 by 105 mm. cuvette|| is filled with distilled water as a reference standard, and the galvanometer needle is set to zero optical density on the scale with water in place in the cuvette well, using a wave length of 500 mm., the point of maximum light adsorption by the red-orange solution.

The optical density of each test solution is read in turn, and the values are recorded as being directly proportional to the dye left in solution.

*Sodium chloride, N/100 HCl, quinine hydrochloride, ammonium sulfate, magnesium sulfate, caffeine sulfate, morphine sulfate, quinine sulfate, quinine bisulfate, and sodium sulfate.

†One of us (M. G. M.) suggested this compound, as it was felt that there might be a direct relationship of the compound and the malarial sera.

‡Junior spectrophotometer, model 6, Coleman Electric Co.

§National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y.; certified, 0.1 per cent aqueous sol.

||Coleman Electric Co.

It was found that an aqueous solution of Congo red* with a pH of approximately 6.5 gave an optical density reading of 0.390 at a concentration of 1:250,000 and a wave length of 500 mμ. The Congo red concentration in the test was chosen so as to give an optical density reading of approximately 0.400 with normal sera. The actual final dye concentration, provided none was precipitated, would be 1:210,000. Such a solution theoretically should possess an optical density of 0.465. This figure has yet to be attained. A dye-protein-quinine complex precipitate has always been obtained in small amounts, even in nonmalarial serum.

Tenth normal NaOH was found to be necessary to clear the final dilution mixture of a slight opacity which was encountered in some sera. One drop of the hydroxide is added to all tubes, whether opacity is present or not, to keep this added factor of dilution constant. Acid was tried as a clarifying agent but was found to modify the color of the remaining NaR sufficiently to give false and inconstant optical density.

The presence of a very chylous sera was found to have a direct bearing on the optical density. It is suggested in these cases that fasting sera be used in performance of the test.

When serum was inactivated at 56° C. for from fifteen to thirty minutes, the samples were always found to approach normal readings. Duplicate samples of sera from acute cases, inactivated and noninactivated, were tested. The non-inactivated sera gave significantly low optical density readings, while inactivated sera were found to give no variation from the normal curve, whether the sera were from patients with or without malaria.

EXPERIMENTAL

Monkey Malaria.—Since the preliminary work showed optical differences between malarial and nonmalarial sera using the previously described dye-protein test, it was felt that this test could be used to follow the clinical course of the disease. An experiment with *P. knowlesi* infected *Macacus mulatta* monkeys was set up in an attempt to correlate the dye-protein results before, during, and after drug administration with simultaneous blood film examinations. Control readings on normal sera were taken on all monkeys for several days before infection, and on three of the monkeys seven readings were obtained over a period of nine days. Six monkeys were infected by intravenous inoculation of heavily parasitized *P. knowlesi* blood. The course of the disease was followed by daily thick and thin smears, and dye-protein determinations were made on sera at varying intervals. Smears were taken from peripheral blood, while from 3 to 4 c.c. of blood were drawn from the femoral vein for the dye-protein test.†

Results: The results of the dye-protein tests on serum from monkeys infected with *P. knowlesi* are shown in Fig. 1.

*As each lot of dye may vary, it is recommended that concentration-optical density curves be set up in each laboratory and that these data be used to select wave length and dilution ratios.

†Dye-protein reaction time increased to sixty minutes and centrifugation speed to 4,500 r.p.m. when monkey sera was tested.

Optical density readings in Monkey 0 before infection varied from 0.400 to 0.460 in eight serum samples taken over a ten-day period before infection. Three days after infection the optical density reading was observed to be 0.420, but readings fell steadily thereafter to 0.190 on the sixteenth day of infection. This animal was then treated with atabrine HCl. The readings of serum samples tested during the next six days gradually approached normal and remained there in subsequent samples.

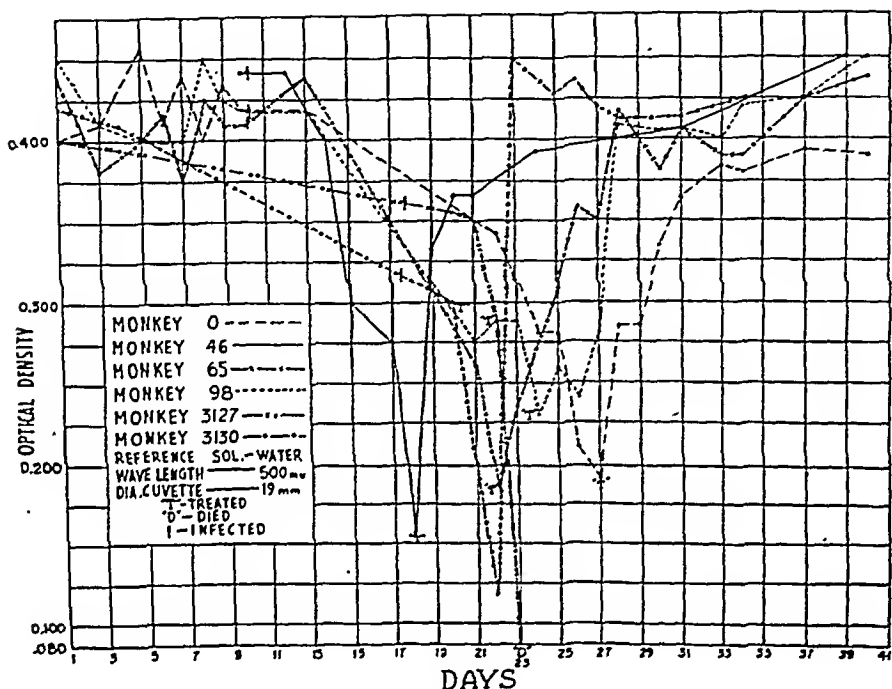


Fig. 1.—Optical density curves of dye-treated sera from monkey infected with *Plasmodium knowlesi*.

Preinfection normal optical density readings of serum samples from Monkeys 46, 65, and 98 varied from 0.380 to 0.450. Only one sample was taken before inoculation of the parasite into Monkey 46. Another serum sample taken two days later from this animal was found to give a similar reading, 0.440. The readings fell gradually during the course of the disease, reaching 0.160 in Monkey 46 on the eighth day of infection and 0.185 in Monkey 65 and 0.285 in Monkey 98 on the eleventh day of infection. After treatment the optical density readings in Monkeys 46 and 65 rapidly approached the normal range within four days, while serum from Monkey 98 dropped to 0.230 two days after treatment was instituted. Four days after further treatment serum samples from this monkey gave normal readings.

Monkey 3127 had the same normal optical density readings as shown for the previously described monkeys but failed to respond to treatment and died. The serum taken immediately before death gave a reading of 0.080.

Chicken Malaria.—The results of the dye-protein test using sera from monkeys suggested further trial using a different parasite and host. Chickens furnished the most economical and convenient host.

Four-week-old chicks were infected with *P. gallinaceum*. One-fourth eubic centimeter of heavily parasitized blood was given intravenously. Control blood levels were taken on all chickens before infection and also on a small group of noninfected birds to ascertain if daily bleedings of from 2 to 3 c.e. were detrimental to the animal or caused enough changes in the blood to change the test readings significantly.

A group of forty-three birds was used in the experiment. Thirty-seven birds were infected and six were used as normal controls. Blood was obtained by heart puncture. Treatment was instituted when the blood picture and gross physical condition of the individual chicken appeared to warrant it. Five-tenths eubic centimeter of a 3.0 per cent solution, quinine HCl, was given intramuscularly.

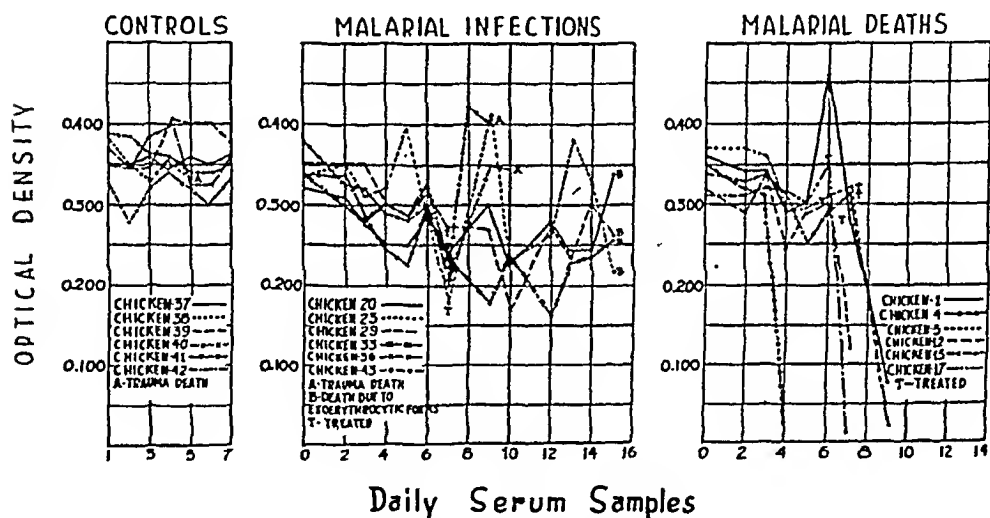


Fig. 2.—Optical density curves of dye-treated sera from chickens infected with *Plasmodium gallinaceum* and controls.

Results: The optical density readings of eighteen chickens are shown in Fig. 2.

In the six control noninfected chickens daily bleedings appeared to have no apparent effect on the blood serum. The normal readings varied between 0.280 and 0.405. One bird, Chicken 41, had consistent low readings varying between 0.280 and 0.340. These findings are depicted in the left side of Fig. 2.

Sixteen of thirty-seven infected chickens died of other causes than malaria. Fifteen of these sixteen chickens died of trauma due to heart puncture, while autopsy on the remaining bird showed pulmonary involvement.

The course of the disease with treatment as interpreted by the dye-protein test is shown in the central portion of Fig. 2. The normal optical density readings of the six chickens correlated with the normals obtained in the control group.

These chickens were infected shortly after the control samples of blood were taken. Four chickens were blood positive the second day after infection, with the remaining two, Chickens 20 and 29, becoming positive on the third day. At that time the readings were gradually falling. On the sixth day there was a decided upswing in the optical density curves of five of the chickens. This same phenomenon was observed in the sixth bird, Chicken 23, on the fifth day. The parasitized red cell percentages on these chickens at the time of this rise in the curves was found to be between 50 and 80. On the seventh day of infection the curves of the six chickens shown in this central graph were observed to fall dramatically, and at this time they were treated. Within twenty-four hours the curve of Chicken 36 rose from 0.210 to 0.420. The optical density curves of Chickens 23 and 43 showed a similar rise in forty-eight hours. The parasite percentages in the latter two were less than 5, and in Chicken 36 it had dropped to 40 from 70 the preceding day. Chickens 36 and 43 died of trauma on the ninth and tenth days. The curve of Chicken 20 was observed to rise from 0.240 to 0.300 forty-eight hours after treatment, falling again with a gradual rise until the day of death. The curve of Chicken 29 was observed to fall gradually until the tenth day at which time the optical density reading began to approach 0.300. Chicken 33 continued to have low readings until the twelfth day at which time there was an upswing toward the normal range. The parasite percentages of these chickens were less than 5 with the exception of Chicken 20 which had had approximately 50 per cent parasitemia throughout the course of the disease. On the fifteenth day of infection Chickens 20, 23, 29, and 33 died. Subsequent brain smears on these birds showed the presence of exoerythrocytic forms of the malaria parasite.

The right side of Fig. 2 depicts the optical density curves of chickens dying before or at the time treatment was instituted. The curves of the chickens in this group follow a similar pattern to those described in the central portion of Fig. 2. There is a general upswing in the curves with a dramatic drop in the next day or two, whether the chickens were treated or not. All birds in this group died of the blood phase of the disease.

Human (Vivax Malaria).—The shape of the optical density curves in the experimental disease in monkeys and chickens suggested that the dye-protein test would serve as a diagnostic aid in human malaria. It was also thought that response to treatment might be followed with the test.

A minimum optical density of 0.340 and a maximum of 0.460 with a mean of 0.390 has been found on twenty-five of nonmalarial human controls without malaria. As no attempt is being made at this time to report on a large series of patients, it is felt that the curves on the sera of seven patients with recurring vivax malaria will demonstrate the principle of the test. Several of the patients have been followed as long as from two to eight months.

Fig. 3 shows the dye-protein curves of human patients with malaria over a period of fifteen weeks. The first blood drawn from the dye-protein test is taken during or shortly after a relapse. This procedure was followed in all patients.

The first optical density reading obtained on Patient 25 was 0.240 with the blood being drawn shortly after a relapse. Within one week the curve had approached 0.330 and remained approximately at that level until the fifth week. The curve gradually declined until the eighth week at which time the patient had another relapse, and a reading of 0.185 was obtained. Three weeks later the optical density curve had reached 0.350.

Patient 26 gave an original reading of 0.240 during a relapse. In two weeks the curve had reached 0.340 and remained above 0.350 throughout the period of thirteen weeks that this patient's serum was tested. During this period the patient had no relapses, and no parasites were observed on thick smears.

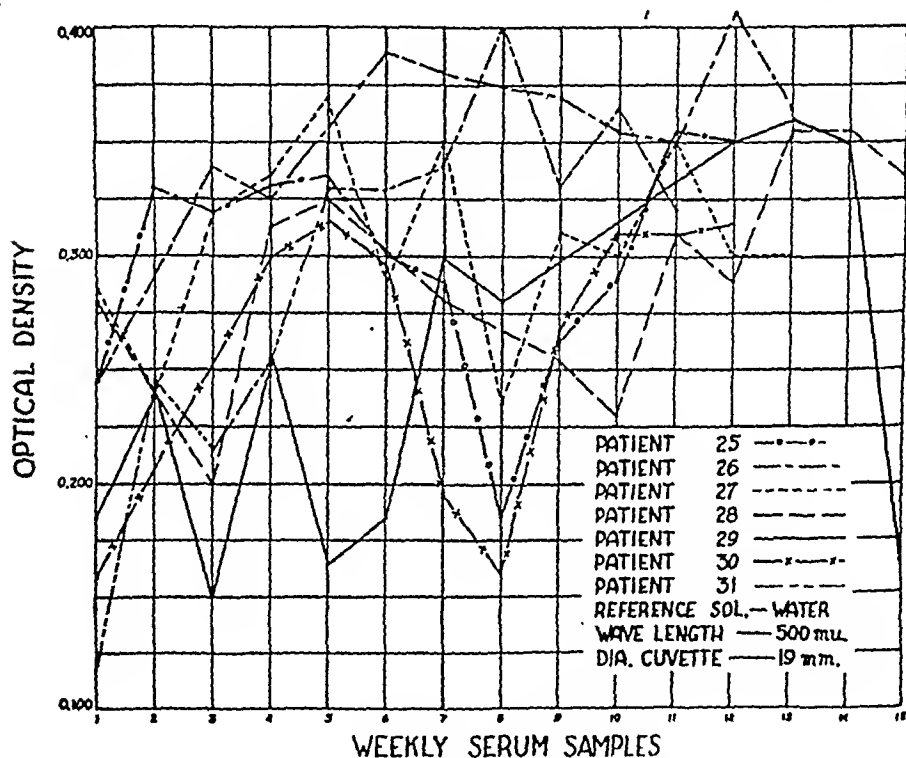


Fig. 3.—Optical density curves of dye-treated sera from human patients infected with *Plasmodium vivax*.

The original optical density reading and all others except the last recorded on the graph were made on Patient 27's serum at other times than during an acute relapse. Health records show eight relapses, while the patient states that he had twenty-nine relapses. A constant parasitemia was observed by blood smear. The readings obtained on serum from this patient over a thirteen-week period ranged between 0.300 and 0.350. Although this patient was in constant parasitemia, only a few parasites were found at each examination.

The original reading from Patient 28 was obtained during subjective symptoms. The optical density curve dropped to 0.200 two weeks later during a clinical relapse. The curve then gradually rose to 0.325 in the next two weeks but

started another gradual decline reaching a low point of 0.230 in the tenth week during a relapse. This patient's curve again rose to 0.350 in the next three weeks and remained there during the follow-up period. Blood smears were positive at the time of clinical relapses.

The first optical density reading of Patient 29 was 0.190 and was obtained during a relapse. This patient also had a constant parasitemia as shown by thick smear. After the original relapse as followed in this study, he relapsed three weeks later giving a reading of 0.150 from a 0.240 rise during the second week. Two weeks later there was another dramatic fall in the optical density curve to 0.165 from 0.255. From this low reading during the fifth week, there was a gradual rise reaching 0.360 in the thirteenth and fourteenth weeks. The following week this patient had another relapse with the curve dropping to 0.165. From the seventh to the fourteenth week the blood smears on this patient were negative, becoming positive at the time of the last recorded relapse on Fig. 3. This patient stated that he had had thirty relapses with an official record of only four in his health record.

Patients 30 and 31 showed very low optical density readings (0.165 and 0.090) during the first relapses to be recorded in this study. There was a gradual rise of both curves to 0.300 or greater with the readings on Patient 31 going as high as 0.400 without further relapses being observed during the study. Patient 30 relapsed in the eighth week with an accompanying lowered reading of 0.160. The optical density curve on this patient again rose to 0.300 and remained at this level during the study. Positive blood smears were observed during the relapse periods of these patients.

DISCUSSION

The present investigation brings into prominence several possibilities of explanation which cannot be further studied at the present time. It was noted, for example, that the quantity of serum used in the test apparently produces no effect upon the optical density readings providing the dye concentration remains unaltered. Variations in quinine hydrochloride concentration produce changes in the readings which are reproducible. Quinine hydrochloride alone will precipitate NaR from solution, while serum and NaR alone give no apparent reaction. At first it was thought that serum acted as a protective colloid in modifying the action of quinine hydrochloride on NaR, but the unchanged readings using variable amounts of sera seem to preclude this theory. It is possible that the A/G ratio is important in the reaction, especially since $(\text{NH}_4)_2\text{SO}_4$ precipitated globulin produced an effect similar to whole serum in the test, while $(\text{NH}_4)_2\text{SO}_4$ precipitated albumin showed no comparable change. It is also possible that the reaction is a combination of globulin denaturation by quinine hydrochloride, A/G ratio, and direct action of quinine hydrochloride and NaR. Somewhere in the interpretation of the test must be considered the drastic changes produced by inactivation of the sera at 56°C . Also, CO_2 frozen sera upon thawing gave optical density readings which did not correspond to results obtained with untreated sera. No correlation of this data has been attempted.

It is possible that the reaction herein described bears no specific relationship to a malarial infection but has as its basis the presence of certain denaturated proteins resulting from the response of the parasite or host, singly or in combination. However, controls other than serum from patients with infectious hepatitis or marked anemia showed no similar reactions.

Although the chemoimmunologic basis of the test is not as yet understood, it is believed that the principle and technique described could be elaborated upon and might possibly be utilized as a significant diagnostic aid in malaria. With the use of different dyes and enhancers, modifications of concentrations, and techniques or other possible changes, it might be possible to make this dye-protein reaction applicable to other diseases.

SUMMARY

A dye-protein test involving the use of Congo red, quinine hydrochloride, and serum has been described in which it is possible to detect serum changes in malarial infections. Observations on experimental and human infections lead one to believe that the test could be utilized as a diagnostic test for latent infections. The technique of the test is not presented as a definitive procedure but rather as one that has sufficient promise to warrant further exploration.

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HEBERDEN'S NODES

V. THE ASSOCIATION OF HYPERTENSION AND OBESITY TO DEGENERATIVE JOINT DISEASE OF THE FINGERS

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HEBERDEN'S nodes are enlargements of the terminal joints of the fingers due to degenerative joint disease. Since numerous observers have noted that degenerative joint disease and hypertension have occurred together, the present study was planned to investigate the blood pressure of women with Heberden's nodes and to determine whether or not this particular form of degenerative joint disease was associated with high blood pressure.

Swett¹ noted the association of arterial hypertension and chronic arthritis. Archer² studying the menopause type of degenerative arthritis found that six of his twenty patients had blood pressure over 150 mm. Hg. Bick³ in a study of 200 cases of chronic arthritis said that the blood pressure in osteoarthritis in most cases is considerably above normal for each age group. O'Reilly⁴ found hypertension in twenty of fifty-six cases of osteoarthritis. Weber⁵ found an average blood pressure of 142 mm. Hg in twenty-one cases of hypertrophic arthritis compared to 127 mm. in atrophic arthritis. Fletcher⁶ in 103 cases of osteoarthritis found obesity, hypertension, and raised erythrocyte sedimentation rate as associated characteristics. He found that a significant proportion of his patients, 36 per cent of those of normal weight and 52 per cent of those with obesity, had hypertension. Most recent students of arthritis have ignored the question.

Heberden's nodes are a particular manifestation of degenerative joint disease which involves the terminal finger joints. Many writers have stressed the fact that Heberden's nodes are typical of degenerative joint disease and are the most common manifestation of this disease. Previous studies⁷ indicate that Heberden's nodes occur frequently as the only significant evidence of degenerative joint disease, that heredity⁸ is an important factor in their occurrence, and that their development depends also to a large degree upon age and sex.⁹

The present study is based on 112 women with idiopathic Heberden's nodes who were seen on the medical service at City Hospital or in private practice. The diagnosis of idiopathic Heberden's nodes as distinguished from traumatic Heberden's nodes depended upon enlargement and deformity of at least three fingers on both hands occurring without relation to trauma. After the patients of this series had been assembled, they were arranged in order of age and divided into groups by decades. Patients were considered to have an abnormally high blood pressure if they had a systolic pressure of

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TABLE I

HEBERDEN'S NODES SERIES 112 WOMEN												CONTROL SERIES 92 WOMEN					
AGE	NUM- BER	AVER- AGE AGE	AVER- AGE B.P.	RANGE B.P.	STAND- ARD DEVI- ATION	NUM- BER WITH HIGH B.P.	PER CENT WITH HIGH B.P.	AGE	NUM- BER	AVER- AGE AGE	AVER- AGE B.P.	RANGE B.P.	STAND- ARD DEVI- ATION	NUM- BER WITH HIGH B.P.	PER CENT WITH HIGH B.P.		
30 to 39	3	37.0	126	120 to 140	± 9.4	0	0	30 to 39	10	35.0	115	100 to 130	±10.6	0	0		
40 to 49	21	45.3	145	108 to 228	±26.4	7	33	40 to 49	26	44.5	143	90 to 210	±31.7	7	27		
50 to 59	36	54.6	153	100 to 240	±49.8	16	41	50 to 59	26	54.0	154	108 to 210	±27.9	11	42		
60 to 69	36	63.7	163	120 to 220	±27.4	22	60	60 to 69	18	64.4	157	115 to 218	±26.1	9	50		
70 to 84	16	75.0	188	144 to 240	±26.8	14	88	70 to 84	12	73.0	163	122 to 205	±25.2	6	50		
112					±27.2	59	53	92					±27.02	33	36		

over 159 mm. Hg. This designation was chosen regardless of diastolic pressure. Only five patients in the study series and three in the control series would have been added to the high blood pressure group if a diastolic pressure of 90 mm. Hg or over also were accepted.

The limit of 160 mm. Hg was chosen to be liberal because these women were not hospitalized or examined under basal conditions, and many of them were seen only once. Page and Coreoran,¹⁰ quoting Symonds, give normal blood pressure in 150,419 healthy men as never higher than 135 mm. Hg. White¹¹ states that normal systolic pressure ranges from 95 to 145 mm. Hg. Bramwell¹² stresses the fact that there is a range of normal blood pressure and that 150 mm. Hg or less is normal. Robinson and Bruceer¹³ state that the normal range of blood pressure is from 90 to 120 mm. Hg systolic. Hines considered 160/100 mm. Hg the upper limit of normal.

The patients with Heberden's nodes are compared to a control series of ninety-two women chosen previously so as to simulate closely in age and sex distribution the original Heberden's node series studied for heredity.¹⁴ As will be noted from Table I, the two series are quite similar in size and age distribution. The average blood pressures in each age group are nearly the same in both series, being only a trifle higher in the Heberden's nodes series. The range of blood pressures in each group is great. This, and the fact that the series are small, explains why the standard deviation is so great. These differences in blood pressures between the two groups are not statistically significant. Even in the eighth decade where the difference is greatest, the mean blood pressures in the two groups are 188 and 163, less than the standard difference of the series.

The percentage of women with high blood pressure may seem unduly high, but it does not vary widely from those found in the population in general. Master and Marks¹⁵ found the percentage of women with hypertension (systolic pressure over 150 mm.) to be 20 in the forties, 43 in the fifties, 63 in the sixties, and 68 in the seventies. The Metropolitan Life Insurance Company¹⁶ found that 20 per cent of women at 40 years of age had hypertension (systolic pressure over 140 and a diastolic pressure over 90).

Although there was no significant difference in the incidence of hypertension between the affected and the control group, the relationship of hypertension to Heberden's nodes was further tested by combining the two series into one, comparing the incidence of hypertension in the Heberden's nodes and the normal series, and computing the coefficient of association between Heberden's nodes and high blood pressure. This has been done in Table II. The coefficient of association was found to be 0.25.¹⁷ According to the computation used an answer of +1 indicates that the attributes under consideration are invariably associated with each other, neither one occurring independently of the other. When the answer is -1 there is complete negative association and neither attribute ever occurs in association with the other. The result found here, 0.25, must be interpreted as of no significance.

TABLE II. HEBERDEN'S NODES AND HYPERTENSION FOUND IN TWO HUNDRED AND FOURTEEN WOMEN

AGE	NUMBER	HEBERDEN'S NODES AND HYPERTENSION	HEBERDEN'S NODES WITH- OUT HYPERTENSION	HYPERTENSION WITHOUT HEBERDEN'S NODES	NO HEBERDEN'S NODES NO HYPERTENSION
30 to 39	13	0	3	0	10
40 to 49	47	7	14	7	19
50 to 59	62	16	20	11	15
60 to 69	64	22	24	9	9
70 to 79	28	14	2	6	6
Total	214	59	63	33	59

TABLE III. HEBERDEN'S NODES FOUND IN EIGHTY-TWO WOMEN WITH HYPERTENSION.

AGE	NUMBER	NUMBER AFFECTED	INCIDENCE FOR AGE	EXPECTED NUMBER AFFECTED
40 to 49	8	0	0.010	0.08
50 to 59	35	1	0.026	0.91
60 to 69	24	2	0.155	3.72
70 to 79	15	1	0.247	3.70
	82	4		8.41

TABLE IV. HEBERDEN'S NODES AND OBESITY IN ONE HUNDRED AND EIGHTY-FOUR WOMEN

AGE	NUMBER	HEBERDEN'S NODES AND OBESITY	HEBERDEN'S NODES WITHOUT OBESITY	OBESITY WITHOUT HEBERDEN'S NODES	NO HEBERDEN'S NODES NO OBESITY
30 to 39	13	1	2	3	7
40 to 49	40	6	8	11	15
50 to 59	50	7	17	12	14
60 to 69	53	11	24	5	13
70 to 79	28	4	12	1	11
Total	184	29	63	32	60

As a further test eighty-two women with hypertension were examined on the wards or in the clinic of City Hospital in a systematic search for Heberden's nodes in such a group. Table III shows that four were found, only one-half of normal expectation in a series of this size and age distribution. The expected number affected was compiled from the incidence actually found in a routine observation of nearly 7,000 individuals.⁹ The series is small and too much reliance cannot be placed upon it, but the findings do not support the supposition that Heberden's nodes and high blood pressure are attributes associated with each other in a proportion higher than in the population at large.

The relationship between Heberden's nodes and obesity was then investigated. An association between obesity and degenerative joint disease has been recognized. O'Reilly⁴ found that 12 per cent of his patients with degenerative joint disease were overweight. Weber⁵ in an analysis of 150 patients noted that patients with degenerative joint disease had an average weight 16 pounds greater than patients with rheumatoid arthritis, despite the fact that they were 0.3 inch shorter. Fletcher⁶ found that obesity occurred eight times as commonly in patients with degenerative joint disease as it did in the population in general. Kovacs and Hartung¹⁸ found that fifty patients with degenerative joint disease weighed an average of 20 pounds more than fifty other

patients with rheumatoid arthritis. Haden and Warren,¹⁹ studying fifty patients with degenerative joint disease, found that 62 per cent of the group weighed more than normal for their age, the average overweight being 25 pounds. Matz,²⁰ in 169 ex-service people with degenerative joint disease, found that 45 per cent were more than 5 per cent over standard weight. The women in our series were judged to be overweight if they were 20 per cent or more above the ideal weight for height and age as given in standard tables. Again the Heberden's nodes and control series were combined (Table IV). The coefficient of association of Heberden's nodes and obesity is 0.08 and therefore of no significance whatever.

COMMENT

The validity of this study depends upon the reliability of the observations and the selection of the material observed. The women with Heberden's nodes were selected solely because of the disease of their fingers in a study which took no account of blood pressures. This was random sampling so far as blood pressure was concerned. The control series is composed of the sisters of women who had no arthritis. They were chosen in the course of a previous study to compare the incidence of Heberden's nodes in the sisters of women without arthritis with the sisters of women with Heberden's nodes. The only other selection exercised was aimed to assemble a group with the same age distribution as the study series. No attention was devoted to blood pressure or obesity at the time other than recording these data. Eighteen of the women in the Heberden's nodes series were private patients. The other seventy-four women of the Heberden's nodes series, and all the members of the control series, were dispensary patients. The members of both series had lived in Cleveland and its environs all their lives. In general the people under consideration were in the modest or low income groups, many but not all of whom had done hard work. The two series seemed to be closely matched in all observable qualities except the presence of Heberden's nodes. According to the literature cited there seems to be an association of degenerative joint disease and hypertension. In no instance had the data presented been subjected to statistical analysis to determine the degree of significance of the findings. Furthermore, there was no comment as to whether or not the incidence of hypertension in patients with degenerative joint disease was any higher than that found in the population in general. Several of the studies cited^{15, 16} indicate that it was not. After comparing the data from the literature with the findings of the study, it seems safe to conclude that patients with Heberden's nodes do not differ from those with degenerative joint disease of other joints in so far as the incidence of hypertension is concerned. No significant association exists between the two conditions.

Data from the literature indicates a strong association between degenerative joint disease and obesity. This association has been based upon very small deviations from standard weight, in some instances as small as 5 per cent in one study.²⁰ In two studies^{5, 18} patients with degenerative arthritis were compared to patients with rheumatoid arthritis, a disease frequently

associated with debility and loss of weight. In four studies^{4, 6, 10, 20} comparison was made to standard tables or to the incidence of obesity in the general population. In the present study no difference was observed in the incidence of obesity in Heberden's nodes and the control series. In respect to obesity, patients with degenerative joint disease of the fingers differ widely from patients with degenerative joint disease of other joints.

SUMMARY

A comparison was made between 112 women with Heberden's nodes and ninety-two women of approximately the same age distribution but otherwise selected at random. It was found that average blood pressures for each age decade were the same in each group. After combining the series into one group, no significant association between Heberden's nodes and hypertension could be demonstrated. In a series of eighty-two women with hypertension, Heberden's nodes were not found more frequently than could be expected from the incidence of this disease in the population in general. It is concluded that Heberden's nodes or degenerative joint disease of the fingers is no different, in relation to hypertension, than is degenerative joint disease of other joints. Despite the fact that a strong positive association has been demonstrated between degenerative joint disease and obesity, no such association was found in this series between Heberden's nodes and obesity. In this respect Heberden's nodes or degenerative joint disease of the fingers differs widely from degenerative joint disease of other joints.

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LABORATORY METHODS

THE DETERMINATION OF SULFONAMIDES IN TUNGSTIC ACID (FOLIN-WU) BLOOD FILTRATES

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SIMULTANEOUS determinations of blood urea, nonprotein nitrogen, sugar, etc., and the unconjugated sulfonamides are frequently required in research or are requested in the clinical laboratory. Hence a method for sulfonamides on routine, tungstic acid (Folin-Wu) blood filtrates should be helpful.

In the present paper are described colorimetric and photoelectric adaptations of the well-known Bratton and Marshall¹ procedures to tungstic acid blood filtrates. The conjugated sulfonamides were not investigated. While it was found necessary to study rather extensively many factors relating to these methods, only a few of the more important findings can be reported in detail.²⁻⁴

EXPERIMENTAL

Ordinary routine blood filtrates generally contain tungstate ions. Upon addition of the usual Bratton-Marshall reagents, these ions produce turbidities and also inhibit coupling of the diazotized sulfonamide and the color reagent. However, it was found that citric acid would inactivate these tungstic acid ions, if they are present in but small quantities.⁵ The addition of either p-toluenesulfonic, trichloroacetic, or dilute sulfuric acid was also found necessary in order to stabilize and keep in solution the resultant dye. Under these conditions a deep, clear, rose-red or orange-red solution results.

Reagents.—

Sodium Nitrite: A 0.1 per cent solution is prepared weekly from a 10 per cent solution which seems relatively stable when kept in the cold.

Sulfamate Buffer Solution: Ammonium Sulfamate,* 0.5 Gm., and sodium acid phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), 13.8 Gm., per 100 c.c. of solution.

Color Reagent: N-(1-naphthyl) ethylenediamine dihydrochloride,* 100 mg., are dissolved in 100 c.c. of water and preserved in a dark bottle.

Stabilizing Solutions: P-toluenesulfonic acid monohydrate,† 75 Gm. are dissolved in water and decolorized by boiling with Norit A‡ decolorizing carbon. The filtered solution is diluted to 500 c.c., and 10 Gm. crystalline citric acid are added. After the present work was partially completed, it was noted that the use of p-toluenesulfonic acid is rather expensive for routine hospital work. Two

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*LaMotte Chemical Products Co., Baltimore, Md.

†Eastman Kodak Company, Rochester, N. Y.

‡Pfanstiehl Chemical Co., Waukegan, Ill.

per cent citric acid in $N/5$ H_2SO_4 was then found to constitute a satisfactory stabilizing solution for all sulfonamide determinations studied except sulfathiazole. The dye which sulfathiazole yields upon coupling with this color reagent under these conditions is insoluble, except in the presence of an acid such as p-toluenesulfonic acid or trichloroacetic acid. Hence, for sulfathiazole determinations, the best stabilizing solution found is a 20 per cent p-toluenesulfonic-2 per cent citric acid solution. These solutions are diluted 1 to 1 with water for the photoelectric method.

Standard Sulfonamide Solutions: Sulfanilamide, 1,000 mg., sulfapyridine, 250 mg., sulfamethazine, 250 mg., sulfathiazole, 250 mg., and sulfamerazine, 250 mg., are dissolved in $N/11.4$ H_2SO_4 and then made up to 1,000 c.c. with the acid. While water could be used for these standard solutions, the sulfonamides are more soluble in dilute acid. Furthermore, these solutions were also used in the recovery experiments to be described later in this paper. One hundred milligrams of sulfadiazine is made up to 2,000 c.c. in $N/11.4$ H_2SO_4 . Appropriate working standards are prepared by dilution of accurately measured quantities of the stock solutions with distilled water. In routine work all standards need not be prepared. The values for the various colorimetric or photoelectric readings, except for sulfanilamide, can be obtained from a conversion table or standard calibration curve given by a comparative analysis of the various sulfonamides.

Sodium Tungstate Solution: A 10 per cent solution is carefully prepared by dissolving 100 Gm. in water and diluting to 1,000 c.c. This solution must not be too alkaline. Thus, 10 c.c. of the tungstate solution to which 5 c.c. of $N/10$ HCl has been added should require at least 4.7 c.c. of $N/10$ $NaOH$ for neutralization to phenolphthalein.

PROCEDURES

Colorimetric Adaptation of the Bratton-Marshall Method to Tungstic Acid Filtrates.—One volume of blood is hemolyzed with eight volumes of $N/11.4$ H_2SO_4 .^{*} It is usually well to add an extra drop of $N/1$ H_2SO_4 . If less than 5 c.c. of blood were collected in the oxalate bottle, however, a drop of 5 N H_2SO_4 must be used for hemolysis in addition to the usual amount of $N/11.4$ H_2SO_4 . One must never neglect this precaution. This amount of acid lowers the pH of the filtrate to about 3 to 2.4 and minimizes the escape of tungstate ions. The decrease in pH does not seem to interfere with any of the routine blood chemistry determinations. The blood proteins are precipitated in the usual manner with 10 per cent sodium tungstate.

^{*}In our hospital this slightly stronger acid is used. Instead of the $N/12$ H_2SO_4 . In order to correct for the alkalinity of the potassium oxalate used to prevent blood coagulation. One cubic centimeter portions of a 2 per cent potassium oxalate and 0.3 per cent sodium fluoride solution are evaporated to dryness in small bottles and used for the collection of routine bloods. This amount of anticoagulant is sufficient for from 15 to 20 c.c. of blood. Thus the concentration of oxalate will depend upon the amount of blood obtained from the patient. If small samples of blood (from 1 to 2 c.c.) are withdrawn, the increased concentration of potassium oxalate will neutralize some of the sulfuric acid and consequently will permit an excess quantity of tungstic acid ions to escape into the filtrate. Accordingly, after diazotization and addition of the color reagent, the solution will be turbid and will have a violet instead of a red color. Also, the dye will immediately flocculate. While in research work the correct relation of oxalate to blood (from 1 to 2 mg. per cubic centimeter) can be readily maintained, this is not practical in routine hospital work.

TABLE I. COMPARISON OF COLORS GIVEN BY VARIOUS SULFONAMIDES

SULFONAMIDE	MG. PER 5 C.C.	COLORIMETER READING (MM.) SET AT	COLOR	CONVERSION FACTOR (PERCENTAGE)
Sulfamerazine	0.1	20.0	Rose red	100.0
Sulfathiazolo	0.1	18.5	Rose red (violet tinge)	108.1
Sulfamethazine	0.1	20.8	Rose red	96.15
Sulfapyridino	0.1	19.5	Rose red	102.6
Sulfadiazino	0.1	22.0	Red (orange tinge)	90.9
Sodium sulfadiazino	0.1	24.0	Red (orange tinge)	83.3
Sulfanilamido	0.1	16.7*	Orange red	119.8*
Sulfamerazine	0.05	20.0	Rose red	100.0
Sulfathiazolo	0.05	18.8	Rose red (violet tinge)	106.4
Sulfamethazine	0.05	20.8	Rose red	96.15
Sulfapyridine	0.05	19.6	Rose red	102.0
Sulfadiazino	0.05	21.5	Rose red (orange tinge)	93.0
Sulfanilamide	0.05	15.0*	Orange red	133.3*
Sulfamerazine	0.025	20.0	Rose red	100.0
Sulfathiazolo	0.025	18.9	Rose red (violet tinge)	105.9

* Approximate readings, as these solutions were too orange to match closely.

To 5 c.c. of the filtrate and to 5 c.c. quantities of appropriate standards are added 5 c.c. of the citric-p-toluenesulfonic acid or the citric-N/5 H_2SO_4 stabilizing solution* and 1 c.c. of 0.1 per cent sodium nitrite. After three minutes add 1 c.c. of the color reagent and again mix the contents of the tubes. A deep red color immediately develops and should be stable for at least an hour. The solution should not be turbid nor have a violet off-shade. The standard which most closely matches the unknown is set at 20 mm. in the colorimeter.

Five cubic centimeter amounts of the various standard solutions of the sulfonamides were subjected to analysis in this manner and then compared with each other in the colorimeter. The sulfamerazine preparation was set at 20 mm. The various colors and readings are given in Table I. It was found that the various red colors match each other reasonably well, except sulfanilamide which was too orange red. From the data in this table one can readily obtain the necessary correction factor as compared with sulfamerazine, for example; hence, only a single standard need be prepared, except in the case of sulfanilamide.

Sulfonamide Adsorption by the Protein-Tungstate Precipitates.—Sulfonamide adsorption in appreciable quantities during blood deproteinization is well recognized.^{2, 3, 6} In the various procedures which utilize trichloroacetic acid or p-toluenesulfonic acid as blood protein precipitants, sulfonamide adsorption is minimized by increased acidity of the filtrates and blood dilution up to from 1 to 50.

In the following experiments various factors which could influence sulfonamide adsorption were studied. Five hundred cubic centimeters of blood containing 1 mg. of potassium oxalate per cubic centimeter were obtained from a donor. Five cubic centimeter portions were pipetted into flasks, and sufficient

* While the citric-p-toluenesulfonic acid solution was used in most of the experiments reported in this paper, many of the more vital experiments were checked with the citric-N/5 H_2SO_4 also. The values always checked closely, however. While the color obtained in the latter solutions was slightly more orange red, the actual amount of color was the same and the readings nearly identical. With sulfathiazole, however, clear solutions were obtained only with p-toluenesulfonic acid which dissolves the dye. Trichloroacetic acid can also be used but does not seem as satisfactory. If considerable quantities of tungstate ions are present in the filtrate, due to excess oxalate in the blood, even a large excess of citric acid does not protect against their interference in the reaction.

quantities of the standard sulfonamide solutions in N/11.4 H_2SO_4 were added in order to obtain a series of bloods ranging from 2.5 to 30 mg. sulfonamide per 100 c.c. of blood. Then sufficient N/11.4 H_2SO_4 was added to bring the amount of acid up to 40 c.c. in each case. After adding a drop of N/1 H_2SO_4 , each flask was swirled gently for several minutes. Then 5 c.c. of 10 per cent sodium tungstate were added slowly with shaking. The flasks were shaken for several minutes. The contents were poured onto No. 1, 12.5 cm. Whatman filter papers.* The complete hemolysis, precipitations, and filtrations were carried out at approximately 18, 26, and 33° C., respectively. The filtrates were then analyzed for their sulfonamide content as previously outlined.

The results obtained from these experiments proved that sulfonamide adsorption is not only considerable but varies with the nature of the sulfonamide as well. However, the percentage recovery in the filtrates for any one sulfonamide is consistent and independent of its concentration in the blood. Hence, a factor can be used to obtain the correct value in each case. Thus, the percentage recovery at 26° C. for sulfanilamide is 88.3; sulfadiazine, 75.7; sodium sulfadiazine, 75.8; sulfamerazine, 75.8; sulfamethazine, 76.1; sulfapyridine, 74.4; sulfathiazole, 56.7; and sulfacetimide, 83.8. Similar values are obtained when 2, 3, and 4 c.c. of blood are deproteinized. The recovery values are slightly higher (from 2 to 6 per cent) when the bloods are deproteinized at approximately 33° C. and slightly lower (from 0.5 to 2 per cent) at 18° C. It is obvious that the degree of adsorption does not parallel sulfonamide solubility. Adsorption of conjugated or acetylated sulfonamides was not studied. Other experiments showed that dilution of blood up to from 1 to 40 does not result in complete recovery of the drug. This loss is not due to inhibitory factors in the tungstic acid filtrates nor do sulfonamide-free filtrates yield appreciable color blanks.

Carrier and Osterberg⁷ found that 20 per cent of the sulfathiazole added to bile was adsorbed when filtrates were prepared with potassium hydroxide and zinc sulfate. Davis⁸ has recently shown that the albumin of plasma appreciably binds the sulfonamides. In the present experiments it was also found that the protein content of blood may undergo considerable depletion, up to 35 per cent, before an appreciable decrease in sulfonamide adsorption is observed. In such cases, however, it is well to decrease the sodium tungstate by about 10 per cent to assure satisfactory filtrates. Also, 2 or 3 c.c. portions of sulfonamide-containing bloods were hemolyzed at different pH by adding an extra drop of sulfuric acid of varying strengths. The bloods were then deproteinized and analyzed in the usual manner. It was found, however, that but a slight decrease in sulfonamide adsorption (from 2 to 6 per cent) occurs as the pH of the filtrates drops from about 3.7 to 2.4.

*Several varieties of Whatman filter paper manufactured by W. & R. Balston, Ltd., England, including the acid treated No. 30 and 40 types and the semicrimped, qualitative filter paper made by Fisher Scientific Co., Pittsburgh, Pa., were found satisfactory. All yielded the same percentage sulfonamide recovery values. Likewise blood filtrates obtained by centrifugation also contained the same concentration of sulfonamides. However, the Reeve Angel's filter paper (H. Reeve Angel & Co., New York, N. Y.) was found quite unsatisfactory. Filtrates obtained with this paper, when subjected to analysis, always yielded turbid solutions and a violet off-shade.

TABLE II. RELATION OF COLOR INTENSITY TO AMOUNT OF SULFONAMIDE (CALIBRATION CURVES)

AMOUNT OF SULFONAMIDE (μ G PER 2 C.C.)	READINGS ON THE KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER FOR VARIOUS SULFONAMIDES WITH THE MODIFIED BRATTON-MARSHALL PROCEDURE					
	SULFA- NILAMIDE	SULFA- THIAZOLE	SULFA- PYRIDINE	SULFA- DIAZINE	SULFA- MERAZINE	SULFA- METHAZINE
50.0	801	520	551	550	520	484
25.0	398	268	280	274	265	243
12.5	201	136	141	140	133	123
6.25	101	69	71	70	66	63
3.125	50	34	36	36	33	31
1.563	24.5	17	18	17	16	16

Photoelectric Adaptation of the Bratton-Marshall Method to Tungstic Acid Filtrates.—Two cubic centimeters of the filtrate, 2 c.c. portions of appropriate standard sulfonamide solutions, and 2 c.c. of distilled water are each diluted with 5 c.c. of the citric-p-toluenesulfonic acid or the citric-N/5 H_2SO_4 stabilizing solution (which has been diluted 1 to 1 with water). Add 0.5 c.c. of 0.1 per cent sodium nitrite. After three minutes add 0.5 c.c. of the sulfamate buffer solution. Mix, wait for three minutes, and add 0.5 c.c. of the color reagent. A deep red color develops immediately and remains clear and stable for at least an hour. Turbidity and a violet off-shade with consequent low readings indicate an excess of tungstic acid ions in the filtrate. The instrument is adjusted to zero reading with the water blank. The readings in the present experiments were made about three minutes after color development in a Klett-Summerson photoelectric colorimeter. It contained a green No. 54 filter which came with the instrument.

TABLE III. COMPARATIVE ANALYSIS OF SULFONAMIDE-CONTAINING BLOODS (MG. PER 100 C.C.)*

NUMBER	SULFONAMIDE	BRATTON- MARSHALL COLORIMETRIC PROCEDURE	ANALYSIS OF TUNGSTIC ACID BLOOD FILTRATES BY THE MODIFIED BRATTON-MARSHALL METHODS		HOSPITAL VALUES BY PHOTOELECTRIC BRATTON-MARSHALL METHOD
			COLORIMETRIC	PHOTOELECTRIC	
1	Sulfadiazine	28.5	28.9	29.6	28.0
2	Sulfadiazine	12.0	11.6	11.5	11.9
3	Sulfadiazine	9.0	8.9	8.7	8.7
4	Sulfadiazine	3.6		3.2	3.1
5	Sulfadiazine	1.1	1.3	0.8	1.0
6	Sulfathiazole	52.2	53.5	48.3	51.0
7	Sulfathiazole	24.0	24.1	24.6	21.0
8	Sulfathiazole	13.2	13.6	14.2	12.5
9	Sulfathiazole	6.0	6.5	6.5	
10	Sulfathiazole	3.2	2.8	2.9	3.2
11	Sulfapyridine	19.5	21.5	18.9	20.0
12	Sulfapyridine	9.6	10.3	9.3	10.0
13	Sulfapyridine	3.0	3.3	3.0	3.0
14	Sulfapyridine	1.7	1.4	1.9	1.5
15	Sulfamerazine	16.7	18.7	16.8	17.5
16	Sulfamerazine	10.5	10.6	10.1	10.7
17	Sulfamerazine	7.9	7.6	7.5	7.3
18	Sulfanilamide	19.0	18.8	19.0	19.4
19	Sulfanilamide	9.4	9.4	9.0	9.5
20	Sulfanilamide	3.2	3.3	3.1	3.5

*Individual standards were used for each sulfonamide determination. The values obtained with the methods on tungstic acid filtrates were corrected for sulfonamide loss during deproteinization.

The blood values may also be obtained from a calibration curve obtained by analysis of a series of standard sulfonamide solutions as previously described. The data for a number of the more common sulfonamides, given in Table II, indicate that the relationship between the concentration of the various sulfonamides and the intensity of color is linear between 2 and 50 μg per 2 c.c. of solution. By proper correction for sulfonamide loss during deproteinization, values expressed as milligrams per 100 c.c. of blood may be obtained directly from the Klett-Summerson readings.

Comparative Analysis of a Series of Sulfonamide-Containing Bloods.—A number of bloods from patients undergoing sulfonamide therapy were obtained from the hospital laboratory. These bloods had been previously analyzed by the regular hospital laboratory staff, using the well-known Bratton-Marshall¹ photoelectric procedure. They were then analyzed by the new methods as adapted to tungstic acid filtrates and by the regular Bratton-Marshall¹ visual colorimetric procedure. The results are given in Table III and indicate that the values given by the various methods are in satisfactory agreement.

SUMMARY

The colorimetric methods of Bratton and Marshall¹ for blood sulfonamides have been adapted to tungstic acid (Folin-Wu) blood filtrates. Various factors which influence these methods have been studied. Comparative analyses of bloods from patients undergoing sulfonamide therapy were made by various procedures, and the values obtained were found to be in satisfactory agreement.

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A SIMPLY CONSTRUCTED METABOLISM CAGE FOR SMALL RATS

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IN CONNECTION with studies of the metabolism of some of the vitamins of the B group, it was necessary to collect urine quantitatively from a small rat, in such a way that there was no contamination of the urine by feces or food. It was also necessary to be able to measure the actual food consumption, either with ad lib. or with restricted feeding, and to feed small known amounts of food to the rat in such a way that all the food given could be consumed.

A Hopkins metabolism cage,* modified in such a way as to allow the rat to obtain small amounts of food quantitatively, was found to fulfill all these requirements.

The metabolism cage is illustrated in Figs. 1 and 2. As shown in Fig. 2, the cage proper, *A*, fitted with a feeding tube, *B*, and a water bottle, *C*, rests in a stemless filter funnel, *D*. A beaker, *E*, containing a small Erlenmeyer flask, *F*, fitted with a conical glass bulb, *G*, is placed so that the tip of the bulb is directly beneath the funnel.

The cage is made of wire or tin and may be of any appropriate size depending on the size animal to be used in it. The floor is made of 22 gauge galvanized wire mesh soldered on to the outside. The lid is made to fit over the outside, and a wire rest is attached for the water bottle, *C*, the glass tube from which is led through the lid and extends a few inches into the body of the cage. The hole cut out of the side of the cage for the feeding tube is $1\frac{1}{2}$ inches in diameter, with the center approximately $1\frac{1}{4}$ inches from the floor. A copper wire about 10 inches long and bent so as to form a circular support for the feeding tube is soldered to the cage on both sides of this hole.

The feeding tubes, *B*, which are made from test tubes, are in two sizes: size A, from 0.94 to 0.95 inches in internal diameter, approximately 3 inches long; size B, from 1.08 to 1.12 inches in internal diameter, approximately 4.5 inches long.

The tubes are flanged as shown in Fig. 2 and are clamped in position by means of the wire support which can readily be bent into shape with the fingers. The tube must be held firmly.

The diameter of the opening of the funnel should be at least $\frac{5}{8}$ inches to prevent blockage by solid material. The bulb, *G*, which is blown from soft glass tubing, is about $1\frac{1}{2}$ to 2 inches in diameter and 2 inches high, with a stem from 1 to $1\frac{1}{2}$ inches long fitting into indentations made in the neck of a 100 c.c. Erlenmeyer flask, *F*. The stem is ground into the indented neck of the

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flask by means of emery powder and glycerin; in this way, the bulb is attached rigidly to the Erlenmeyer flask. The indentations in the neck allow the urine to run into the flask.

The distance between the funnel and the top of the bulb should be just sufficient to allow feces to fall away; if it is too great, liquid tends to splash off the bulb. The shape of the bulb is most important; it should be peaked, with

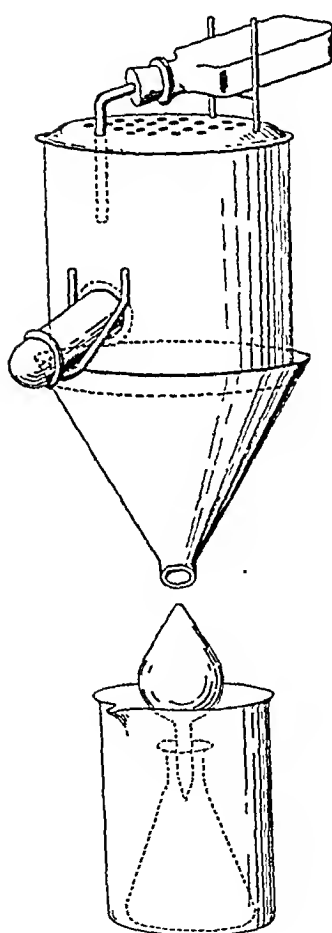


Fig. 1.

Fig. 1.—Assembled cage.

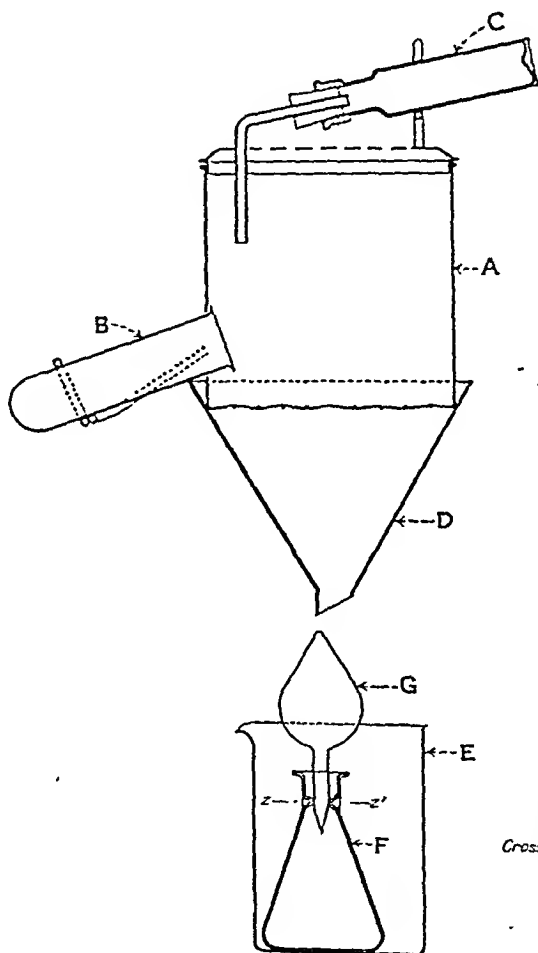


Fig. 2.

Fig. 2.—Cross section of assembled cage through central axis.

steep but not vertical sides which should merge gradually into the stem so that liquid runs down the stem and does not drip from the bulb.

The dimensions of the feeding tubes should be such that the rat can reach the bottom of the tube, but only with difficulty. With such a tube, the rat is unable to remove food from the tube with its paws; if the tubes are made larger,

the rats invariably shovel the food into the cage with their front paws. It was found that size A was suitable for rats weighing from 20 to 40 grams, and size B was suitable for rats weighing from 40 to 80 grams. A larger feeding tube is necessary for rats weighing more than 80 grams.

In this type of cage, the nature of the food mixture given to the rats is important. If a dry powdery mixture is used, considerable scattering occurs, occasionally causing blockage of the funnel. To prevent this, such a powder should be mixed with water to form a thick mash. More suitable types of food mixtures are those prepared from sugar, oils, and casein. The large particle size of the sugar and the binding action of the oil both tend to prevent scattering. A mixture with a high fiber content is undesirable, because the bulk of the feces formed sometimes causes blockage at the mouth of the filter funnel.

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AN IMPROVED METHOD FOR THE DETERMINATION OF PLASMA PROTHROMBIN

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INTRODUCTION

MANY methods have been employed for the clinical determination of the prothrombin concentration of plasma. All of these methods are based upon the principle that in the presence of an optimum calcium ion concentration and an excess of thromboplastin the coagulation time of the plasma is related to the prothrombin concentration.

This relationship, however, has been found to be hyperbolic rather than linear, so that the coagulation time is an insensitive measure of prothrombin concentration when the latter is high but a sensitive measure when it is low. For this reason the original Quick method, which employed undiluted plasma, can be considerably improved in accuracy by dilution of the test plasma with prothrombin-free plasma.

It was the purpose of the present investigation to develop a satisfactory diluent for plasma, a stable thromboplastin preparation, and a procedure for the determination of prothrombin to be used for clinical and experimental purposes. Although this investigation was carried out using dog's plasma, the same procedures can be applied to human plasma.

METHODS

Preparation of Stable Thromboplastin.—In all the methods used to determine prothrombin concentration of blood, the coagulation time is notably influenced by the activity of the thromboplastin suspension. A stable and uniform preparation of this reagent is therefore necessary to render a technique or method reliable.

The concentration of the thromboplastic substance in the brains of different animals of the same species is variable; for example, when three different powdered brains of the same animal species were tested against the same dilution of dog's plasma, the resulting coagulation times were 40, 50, and 32 seconds, respectively.

Quick prepares his thromboplastin by shaking brain powder in normal saline solution and then centrifuging it or allowing it to settle. The supernatant, which is the reagent to be used, has a variable activity because of the influence of temperature, intensity of shaking, and completeness of settling or centrifuging.

It was found that these difficulties could be avoided by employing a suspension of the total brain powder made without centrifuging.

From the Laboratory of Pathology and Experimental Surgery, University of Buenos Aires.
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Preparation of Rabbit Brain Powder: The rabbits are killed by a 20 c.c. intravenous injection of air. Then the meninges are carefully removed and the brains repeatedly triturated with anhydrous acetone in order to rapidly remove all traces of water. The acetone is evaporated at room temperature, and the drying is finally completed in vacuo over sulfuric acid. The dehydrated brain tissue (from 50 to 100 Gm.) is finely pulverized in a mortar so as to obtain a uniform powder. This powder is placed in glass ampules in 0.15 Gm. fractions; before sealing, the ampules are placed in the desiccator in order to remove humidity that may have been absorbed during the weighing process. They are then closed in an atmosphere of nitrogen. Brain powder prepared in this way retains its activity for as long as five months.

Preparation of the Powdered Brain Suspension (Thromboplastin): The contents of one ampule (0.15 Gm. of brain powder) are again finely pulverized in a mortar. By adding 0.3 c.c. of a 0.85 per cent sodium chloride solution, a homogenous paste is obtained and is diluted by four successive additions of 2 c.c. of sodium chloride solution. The dilution is brought up to 50 c.c. by normal saline solution, and the suspension is then shaken and heated for ten minutes at 37° C. to obtain a uniform temperature throughout. The suspension must be agitated by shaking or by blowing down the pipette before taking a sample.

TABLE I. INFLUENCE OF CONCENTRATION OF BRAIN POWDER ON COAGULATION TIME

BRAIN POWDER (GM./100 C.C.)	COAGULATION TIME OF DOG'S PLASMA (SEC.)			
	DILUTION			
	1/0	1/10	1/20	1/50
0.6	10.5	23.5	34.5	78.5
0.3	10.5	18	28.5	40
0.15	12	19	27.5	47
0.075	16	23.5	32	57.5

TABLE II. INFLUENCE OF TEMPERATURE ON ACTIVITY OF 0.3 PER CENT BRAIN POWDER SUSPENSION

TEMPERATURE (° C.)	COAGULATION TIME OF DOG'S PLASMA (SEC.)			
	DILUTION			
	1/0	1/10	1/20	1/50
37	11.5	16	26.5	36
45	12	18.5	26.5	40.5
60	22	36.5	51	83
100	23.5	38	55.5	91.5

The suspension prepared in this way has 0.3 Gm. of powdered rabbit brain in 100 c.c. of the suspension in contrast to Quick's preparation which contains the supernatant from 3 Gm. of brain powder in 100 c.c. of solution.

We have found that a higher concentration of the brain powder considerably prolongs the coagulation time (Table I). This was first shown by Aggeler and Lucia. The mechanism of the anticoagulant effect of an excess of thromboplastin is not understood. We also have found that a temperature of 37° C. is the most convenient for obtaining an active, stable, and uniform suspension. A higher temperature prolongs the coagulation time as can be seen in Table II.

This confirms the results obtained by Quick relative to the influence of the temperature on the activity of the thromboplastin suspensions.

Stability of the Thromboplastin: Stock rabbit brain powder prepared by the previously described method has been found to retain its full activity for periods as long as five months. Table III shows the results of periodic assays of such a preparation. The assay consisted of using the powder to determine the plasma prothrombin level of normal dogs maintained under uniform conditions and used for no other purpose. The results reveal random fluctuations in prothrombin level but no trend in a downward direction indicative of progressive loss of activity of the thromboplastin preparation. The random fluctuation is due, in large part, to the use of normal dog plasma as a source of prothrombin; however, had a uniform preparation of pure prothrombin been available, the random fluctuation could have been minimized.

TABLE III. STABILITY OF THROMBOPLASTIN SUSPENSION TESTED ON PLASMA OF CONTROL DOGS

DOG 133		DOG 134	
DAYS	PROTHROMBIN (%)	DAYS	PROTHROMBIN (%)
1	83	1	108
2	87	2	98
3	81	3	107
4	88	4	94
5	97	5	95
7	100	7	105
10	86	12	114
15	95	17	105
20	90	21	101
25	90		
26	104		
29	92		
36	95		
45	90		
Average	88.4	Average	103
Range	81 to 104	Range	94 to 114

Development and Preparation of a Satisfactory Diluent.—A satisfactory diluent for plasma in the determination of prothrombin must not interfere in any way with the coagulation of the plasma which it dilutes. This means that the diluent must contain no prothrombin activity itself, and it must not influence the prothrombin level, fibrinogen level, or pH of the diluted plasma.

The influence on coagulation time and clot formation of alteration in fibrinogen level and pH has been demonstrated experimentally. In Table IV it

TABLE IV. EFFECT OF FIBRINOGEN CONCENTRATION ON COAGULATION TIME

OXALATED PLASMA (C.C.)	OXALATED SERUM (C.C.)	DILUTION	THROMBIN* 1/10 (C.C.)	COAGULATION TIME (SEC.)
0.1	—	1/0	—	—
0.05	0.05	1/2	0.1	17.1
0.02	0.08	1/5	0.1	21.4
0.01	0.09	1/10	0.1	26.3
			0.1	95

Dilutions of $\frac{1}{2}$ and $\frac{1}{10}$ yield friable clots.

*Thrombin prepared according to Eagle technique.

TABLE V. INFLUENCE OF pH ON COAGULATION TIME

TUBE	pH	COAGULATION TIME (SEC.)
		DILUTION 1/20
1	5.40	33.5
2	6.55	21
3	7.15	20
4	7.85	21.5
5	8.2	22
6	9.4	23
7	10.1	No coagulation at 2 min.
8	10.7	No coagulation at 2 min.

TABLE VI. COMPARISON OF PROTHROMBIN ADSORBENTS

SUBSTANCES	pH OF SUSPENSION	pH OF PLASMA	COAGULATION TIME
SO ₄ Ba	7.2	7.8	No coagulation
CO ₃ Ba	8.2	8.3	No coagulation
CO ₃ Zn	7.3	8.3	No coagulation
(OH) ₂ Zn	8.5	8.7	4 min.
(OH) ₃ Al	8.7	8.6	No coagulation
(OH) ₂ Mg	10.1	9.1	19 min.
(CO ₃ O) ₂ Ca	7.2	8.2	No coagulation
(OH) ₃ Fe	-	-	No coagulation
SO ₄ Pb	2.73	7.6	4 min.
Talcum	-	-	20 sec.
Kaolin	-	-	45 sec.

is shown that dilution of plasma with normal serum, which reduces the fibrinogen level, markedly delays the coagulation time of plasma. In Table V it also is shown that beyond the pH limits of from 6.0 to 8.0 coagulation is markedly interfered with. This confirms the previous work of Mertz and Owen.

Normal plasma from which prothrombin has been completely removed would appear to offer the best source of a satisfactory diluent. A number of investigators have used various procedures for removing prothrombin from solution, including adsorption on magnesium hydroxide (Fuchs), aluminum hydroxide (Quick), tricalcium phosphate (Bordet and Delange), and barium sulfate (Dale and Walpole). We made a comparative study of these and other agents with respect to their effectiveness in removing prothrombin from normal plasma and their pH. The substances studied are shown in Table VI. All of them were prepared by precipitation, using a large volume of water, and then they were washed thoroughly. The precipitate obtained was suspended in a small volume of water and tested as an adsorbent of prothrombin. In every instance the procedure was as follows: 0.2 volume of the suspension was added to 1 volume of plasma; the mixture stood for ten minutes at 37° C., after which it was centrifuged for ten minutes at 3,000 r.p.m. The coagulation time of the supernatant was determined by adding 0.2 c.c. of the thromboplastin-calcium mixture to 0.1 c.c. of the supernatant plasma.

Among the substances tested by us, barium sulfate was the most satisfactory because of its neutrality, its almost complete insolubility, and the facility with which it can be removed by centrifuging. Furthermore, barium sulfate is much more active than the other substances tested regarding its ability to prevent coagulation. In fact, a sample of plasma centrifuged after being in contact

for ten minutes, at room temperature, with 5 per cent barium sulfate suspension, does not clot when thromboplastin and calcium are added. The plasma so obtained is completely clear and has the same pH as normal plasma.

The barium sulfate in the dried form also adsorbs prothrombin but yields plasma with some turbidity which is not cleared by repeated centrifuging.

Preparation of a 30 Per Cent Barium Sulfate Suspension: This suspension is obtained from barium chloride and sulfuric acid. Thirty-five grams of crystallized barium chloride are weighed and dissolved in 500 c.c. of water; while shaking, a solution of 11 c.c. of sulfuric acid (specific gravity, 1.84) in 500 c.c. of water is added. This is filtered or centrifuged and then washed with distilled water until there is no further acid reaction to methyl red. The precipitate is suspended in 100 c.c. of distilled water. The adsorbing property of this suspension diminishes if it becomes acid. It may be renewed by washing with distilled water until a neutral reaction is obtained. The original volume is then restored by the addition of distilled water.

Preparation of Diluent Plasma: To 1 volume of oxalated plasma 0.2 of a volume of barium sulfate suspension is added, shaken, and warmed for ten minutes at 37° C. and then centrifuged at high speed (3,000 r.p.m.) and decanted. We call this plasma barium plasma to differentiate it from plasma treated by other adsorbents. It should not coagulate on adding thromboplastin and calcium, for it has no prothrombin. The barium plasma is more efficacious as a diluent plasma than the alumina plasma of Quick. In Table VII is presented the results of an experiment which demonstrates that the coagulation times are shorter by using barium plasma as a diluent as compared with the alumina plasma. This is due to the fact that some aluminum hydroxide remains suspended and adsorbs prothrombin from the normal plasma.

TABLE VII. COMPARATIVE STUDY OF BARIUM PLASMA AND ALUMINA PLASMA

HUMAN PLASMA (C.C.)	HUMAN (DILUTED) PLASMA (C.C.)	COAGULATION TIME USING BARIUM PLASMA AS A DILUENT (SEC.)	COAGULATION TIME USING ALUMINA PLASMA AS A DILUENT (SEC.)
0.02	0.08	25	32
0.01	0.09	35	92
0.005	0.095	57	No coagulation

Apparatus, Reagents, and Technique of Determination.—The apparatus required consists of a water bath, 0.2 c.c. graduated pipettes divided to 0.001, Kahn tubes, test tubes, and a stop watch.

Fig. 1 shows the water bath used by us. This is composed of a Pyrex beaker of 1 L. capacity (1) and a vessel of bronze (2), 6 cm. in height and 5 cm. in diameter with holes at its base to permit the free circulation of water. The tubes are placed in the bronze vessel and kept at a constant temperature. A second perforated bronze vessel (3), 6 cm. long and 1.5 cm. in diameter, is attached to a flexible bronze wire of 3 mm. in diameter; this wire permits the vessel to be placed in any position desired by the operator. The temperature of the bath may be regulated by a flame, the siphon (4), and the thermometer (5).

The test tube (6) which contains the thromboplastin-calcium-mixture and the pipette (7) are kept in the bath at 37° C. in order to avoid any change in temperature of the plasma.

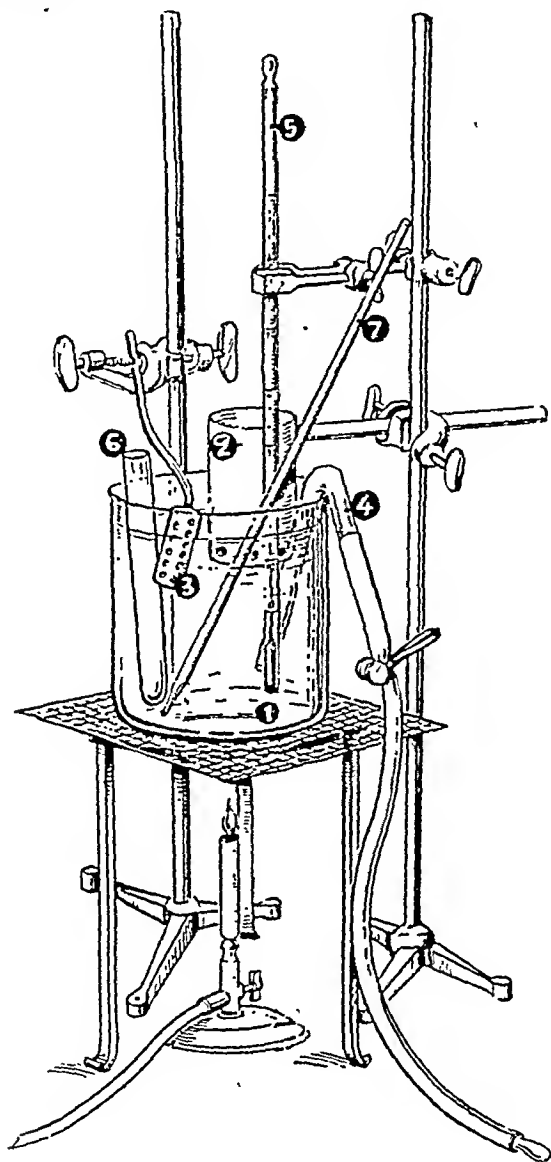


Fig. 1.

The reagents required consist of 0.1 M. sodium oxalate (1.34 Gm. per 100 c.c.), 0.025 M. calcium chloride (0.28 Gm. per 100 c.c.), and the 0.3 per cent thromboplastin and 30 per cent barium sulfate suspensions previously described.

Technique of Determination:

1. Blood extraction. One volume of sodium oxalate is added to 9 volumes of blood. The mixture is centrifuged, and the plasma thus obtained is divided

into two portions. One portion is used in the preparation of barium plasma and the other kept as plasma to be tested.

2. Preparation of the barium plasma (see foregoing).

3. Preparation of thromboplastin-calcium mixture. One volume of thromboplastin suspension is added to 1 volume of 0.025 M. CaCl_2 solution.

4. Different amounts of barium plasma and plasma to be tested are allotted to different test tubes according to the schedule shown in Table VIII.

TABLE VIII

TUBE	P.Ba.* (C.C.)	P.E.† (C.C.)	P.E. 1/10 (C.C.)‡	DILUTION	P.E. % PRO- THROMBIN	THROMBO- Ca (C.C.)	COAGULA- TION TIME (SEC.)
1 + 2	-	0.1	-	1/0	100	0.2	
3 + 4	-	-	0.1	1/10	10	0.2	
5 + 6	0.05	-	0.05	1/20	5	0.2	
7 + 8§	0.05	-	0.02	1/50	2	0.2	

*P.Ba., Barium plasma.

†P.E., Plasma to be tested.

‡The $\frac{1}{10}$ P.E. is prepared by adding 0.05 c.c. of plasma to be tested to 0.45 c.c. of barium plasma.

§When testing human blood 0.07 c.c. of P.Ba. and 0.03 c.c. of P.E., $\frac{1}{50}$ are used, thus giving a dilution of $\frac{1}{52.3}$.

TABLE IX. COAGULATION TIME OF DIFFERENT DILUTIONS OF PLASMA OF NORMAL DOGS

DOG	COAGULATION TIME (SEC.)			
	DILUTION			
	1/0	1/10	1/20	1/50
507	10.5	15.5	26.5	38
509	11.5	16.5	24.5	39
516	11	14.5	25	37
519	10.5	15	23	36.5
520	10	14.5	23.5	36
506	11.5	15	24.5	38.5
508	11	15.5	23.5	37
510	11	14.5	24	38
511	10.5	14	22.5	36.5
512	11.5	15.5	23.5	38
521	11	16	24	36.5
Average	10.9	15.1	24	37.4

The determinations at each dilution are made in duplicate. The barium plasma is always measured out first into each tube to be used so as to avoid possible barium plasma contamination with prothrombin plasma. Then, undiluted plasma to be tested is added and finally diluted plasma to be tested (1/10). Special attention must be paid to keeping the outside of the pipettes dry and to pipetting aliquots into the bottom of the tubes, since small amounts of plasma could remain on the sides of the test tubes. It is also advisable to use a different pipette for each plasma.

The tubes with the measured plasma, the mixture of thromboplastin and calcium, and the 0.1 c.c. pipettes are placed in the 37° C. water bath. Each tube (separately) will then receive 0.2 c.c. of the thromboplastin-calcium mixture, and the coagulation time is determined by means of the chronometer from

the moment the reagent has been added.* The thromboplastin-calcium preparation should be mixed each time by blowing down the pipette before starting the measurements.

The Relationship Between Coagulation Time (Prothrombin Time) and Prothrombin Concentration.—In 1937 Quick and Leu diluted normal human and rabbit plasma with prothrombin-free plasma, prepared by the $Al(OH)_3$ method, in order to obtain various relative concentrations of prothrombin. They were able to show that in the presence of optimum calcium and excess thromboplastin the coagulation time and relative prothrombin concentration bear an inverse hyperbolic relationship to one another.

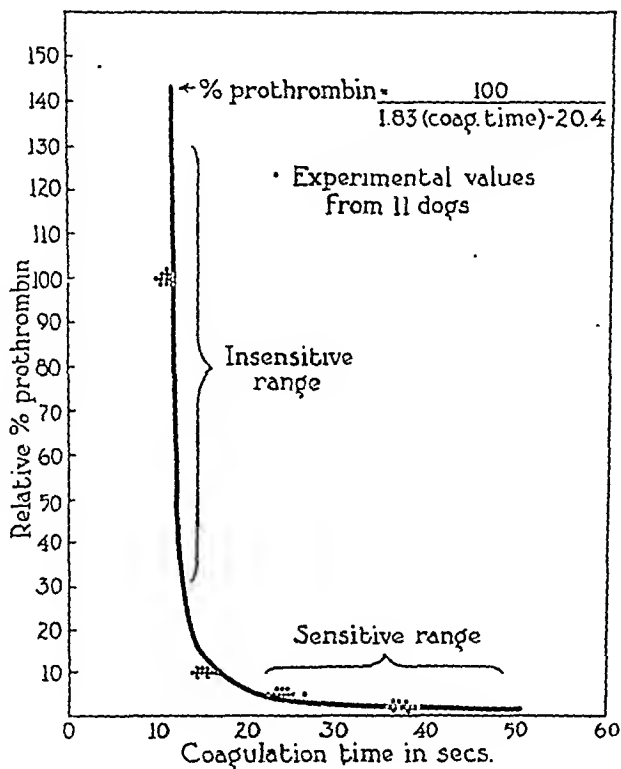


Fig. 2.

These experiments have been repeated using the plasma of eleven normal dogs diluted with prothrombin-free plasma prepared according to the procedure described herein. The percentages of prothrombin and the observed coagulation times are presented in Table IX, and plotted in Fig. 2.

On examining the graph of Fig. 2 it is evident that:

1. A change in prothrombin concentration from 100 per cent to 50 per cent corresponds to a nearly imperceptible change in coagulation time.

*Formation of foam must be avoided in order not to miss the formation of the clot. This is accomplished by adding the thromboplastin-calcium mixture in the following manner: the pipette is placed at the bottom of the tube, and then its contents are blown out; at this moment the stop watch is started and the pipette is removed while continuing the blowing so that the stream of air can break any foam that may have been formed.

2. On the other hand a change in prothrombin from 50 per cent to 2 per cent corresponds to an easily measurable change in the coagulation time.

3. The error due to the measurement of coagulation time is overwhelming if the coagulation time is less than that corresponding to 100 per cent of prothrombin concentration.

Let us consider an example:

- | | |
|--|---------------------|
| 1. A tube with 100 per cent of prothrombin coagulates in 11.7 sec. | } Diff. = 0.5 sec. |
| 2. A tube with 50 per cent of prothrombin coagulates in 12.2 sec. | |
| 3. A tube with 2 per cent of prothrombin coagulates in 38.5 sec. | } Diff. = 26.3 sec. |

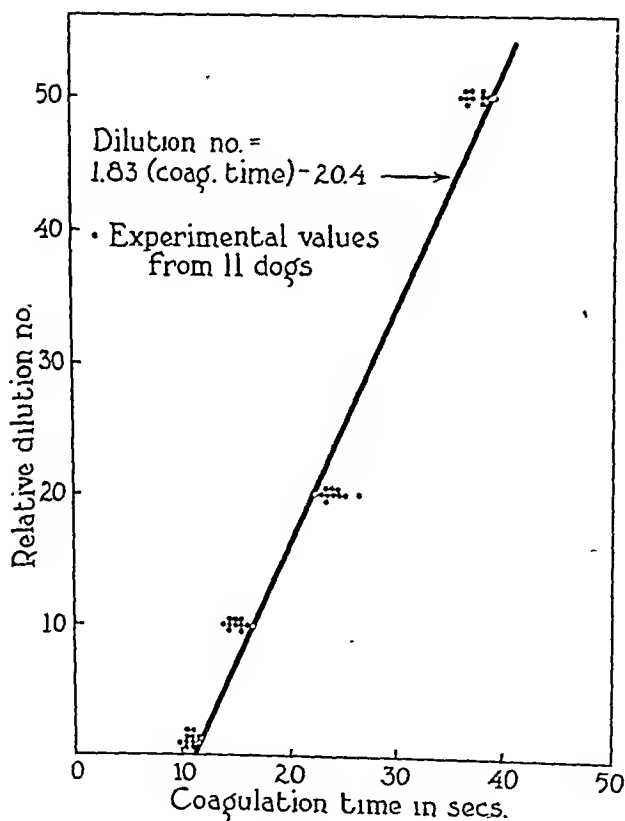


Fig. 3.

The results clearly show that the estimation of concentration using undiluted plasma is inaccurate and that the plasma must, therefore, be diluted in order to reach the sensitive portion of the curve in which significant variation in the coagulation time occurs with small variations in the concentration of prothrombin. For this reason dilutions of 1/10, 1/20, and 1/50 are recommended.

If the relationship between per cent prothrombin and coagulation time, as shown in Fig. 2, is truly a hyperbola, the relationship between the reciprocal of the per cent prothrombin and the coagulation time must be a straight line.

In Fig. 3 $\frac{100}{\% \text{ prothrombin}}$ is plotted against the coagulation time. It can be seen that the relationship is essentially linear.

The equation which best fits the data of Fig. 3 was found to be:

$$\frac{100}{\% \text{ prothrombin}} = 1.83 (\text{coagulation time}) - 20.4 \quad (1)$$

This equation is plotted in Fig. 3 as the straight line. The corresponding equation for the hyperbola plotted in Fig. 2 is obtained by rearrangement of the preceding equation as follows:

$$\% \text{ prothrombin} = \frac{100}{1.83 (\text{coagulation time}) - 20.4} \quad (2)$$

The values of the constants in this equation vary slightly with different stock preparations of thromboplastin and vary considerably for normal plasmas from different animal species. For human plasma the following values have been obtained:

$$\% \text{ prothrombin} = \frac{100}{0.555 (\text{coagulation time}) - 8.24} \quad (3)$$

Calculation of Results: The procedure for the determination of the plasma prothrombin level described herein yields a series of coagulation times for the several dilutions of plasma employed. It is desirable to convert these three figures into a single value representing prothrombin concentration rather than coagulation time. Since the absolute prothrombin concentration is unknown, it must be expressed in terms of percentage of normal. This requires the establishment of normal standards. We have used the following procedure for this purpose. Using a stock preparation of thromboplastin, determination of the coagulation time of many samples of normal plasma at various dilutions are made by the method described. This yields data of the kind presented in Table IX. These data are then plotted as in Fig. 3, for the purpose of obtaining the best fitting linear equation. This equation, when converted to the hyperbolic form, is the standard equation.

When determinations are made on unknown plasmas, the observed coagulation times at the various dilutions are used to calculate the percentage prothrombin from the standard equation. These values are then expressed as a percentage of the standard values at these same dilutions. Expressed in a formula, this becomes:

$$\% \text{ normal prothrombin} = \frac{\text{Observed } \% \text{ prothrombin at a given dilution}}{\text{Standard } \% \text{ prothrombin at the same dilution}} \times 100$$

TABLE X

TUBE	DILUTION	OBSERVED COAGULATION TIME	% OBSERVED PROTHROMBIN	% STANDARD PROTHROMBIN	% NORMAL PROTHROMBIN
1	1/10	18	7.97	10	79.7
2	1/20	24	4.25	5	85.0
3	1/50	46.5	1.54	2	77.0
Average % normal prothrombin					80

If three dilutions are employed, the resulting three values are averaged. An example of such a calculation is shown in Table X, based upon Equation 2 ? as the standard equation.

SUMMARY

1. A method is described for obtaining a preparation of thromboplastin from rabbit brains which retains its activity for at least five months.
2. A method is described for preparing prothrombin-free plasma by adsorption on barium sulfate.
3. Because of the inverse hyperbolic relationship between prothrombin concentration and coagulation time, the accurate determination of prothrombin in plasma requires dilution of the latter with prothrombin-free plasma.
4. An accurate method for determining plasma prothrombin based upon the foregoing findings is described.

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ANTISHEEP AMBOCEPTOR PRODUCTION WITH ELIMINATION OF RABBIT SHOCK

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THE investigation herein described was begun in 1940 at the Division of Serology, Army Medical School, Washington, D. C., and completed in 1941. At that time this Division was making all the serologic reagents for the Regular Army. Therefore, anything hindering quantity production was a serious waster of time.

Chief among the hindrances were the heavy losses of rabbits by shock and, too often, the presence of agglutinogens or precipitins in the finished amboceptor. Everyone who has had to make amboceptor in any large amount knows the considerable losses of rabbits after the second dose of cells. If this dose is made large in the effort to produce amboceptor of a high titer, losses are inevitable. If the second dose is lowered, to escape fatal shock, the amboceptor produced is usually of too low a titer to be serviceable. These faults were inherent in the injection of the hemoglobin fraction of the sheep cells. Since Vedder¹ and Hadjopoulos² had proved that injection of cell stroma after laking would produce amboceptor, we decided to try some modification of their methods which would give a greater yield of cell stroma. The procedure we adopted has been used with perfect success in the Division of Serology for the past five years without the loss of a single rabbit. Since this method has been successfully tested by time, both in our hands and by others, we have felt it wise to offer it for wider use.

Two methods were tried, one by laking the cells with 0.4 per cent salt solution and the other by using amboceptor and complement to attain that end. Both were successful in that they eliminated all signs of shock in the rabbits and produced amboceptor of a high titer in ten days. The amboceptor-complement method produced nearly twice the amount of cell stroma as did the 0.4 per cent saline. Therefore, we adopted the former.

In following the procedure given in the next section, it is stressed that the sheep cells must be processed the same day on which the rabbits are to be injected. The cell stroma does not keep well in the refrigerator. We timed our cell preparation so that we could inject our rabbits immediately, as a rule, but it seemed just as effective to prepare them in the morning and inject them early the same afternoon.

PROCEDURE

Materials.—

1. Fresh washed, packed sheep cells.
2. Complement (pooled serum from twenty guinea pigs).

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3. Amboceptor (titer of 1:2000 or higher).
4. Salt solution, 0.85 per cent.
5. Graduated centrifuge tubes, 15 c.c. capacity.

Method.—

1. To 1.0 c.c. of washed and packed sheep cells add 1.0 c.c. of complement and 0.3 c.c. of undiluted amboceptor.
2. Incubate at 37° C. for fifteen minutes.
3. Dilute to 15 c.c. with 0.8 per cent salt solution, inverting gently once or twice to mix.
4. Centrifuge for five minutes.
5. Draw off supernatant fluid and set it aside.
6. Add 15 c.c. salt solution, mix gently, and combine with the supernatant set aside in step 5. This completes the hitherto incomplete hemolysis.
7. Centrifuge at high speed for thirty minutes.
8. Draw off and discard the supernatant fluid.
9. Add 15 c.c. of 0.85 per cent saline, mix gently, and centrifuge at high speed for thirty minutes. This removes all remaining traces of complement and amboceptor.
10. Draw off and discard the supernatant fluid. The residue of cell stroma is a translucent, pinkish- or light orange-colored mass, only barely distinguishable from the fluid. Care must be taken in all pipetting so that as little cell stroma as possible is wasted.
11. Make up the packed cell stroma to 1.0 c.c. with 0.85 per cent saline and mix gently.

This forms the initial dose for one rabbit. The second dose, given on the fifth day, is 2.0 c.c. The third dose, given on the tenth day, is 3.0 c.c.

It is not advisable to try to handle the sheep cells in amounts larger than 1.0 c.c. at a time. It is found that using larger amounts invariably produces relatively less stroma. As our doses are governed by the amount of original sheep cells, rather than by the final amount of stroma recovered, it is most desirable to recover the maximum yield possible.

The amount of washed and packed sheep cells to be obtained at the start depends, of course, on the number of rabbits to be injected. Thus, if five rabbits are to be treated, 5 c.c. of sheep cells are to be obtained for the first dose, 10 c.c. for the second, and 15 c.c. for the third. Processing fifteen separate centrifuge tubes at a time takes a little longer than processing one alone.

The injection is always made into the marginal ear vein.

Titers of from 1:4,000 to 1:10,000 are obtained after the second dose, that is, on the tenth day. These increase only slightly after the third dose, that is, on the fifteenth day, to from 1:5,000 to 1:12,000. The third dose is rarely needed.

Sex, breed, and age of rabbits apparently play no part in the titer of the amboceptor produced.

Emphasis is laid on the following facts in relation to this method:

1. During five years of use with over 500 rabbits, not a single rabbit has died from shock.

2. Satisfactory amboceptor is produced in ten days, with only a slight rise by the fifteenth day; in either case this is a great saving of time.

3. Titers produced are high compared to those produced by the packed, washed cell method. This is a far from negligible factor, as dilution to an acceptable 1:4,000 strength gives a much greater final yield.

4. It may be thought that the necessary preparation of the sheep cells is an unduly cumbersome and slow process. Actually it takes about ninety minutes, no matter how many tubes are used. This is a negligible factor in consideration of the increased yield and high titer of the product and the saving in rabbits and in time devoted to injection.

5. Finally, amboceptor thus produced gave identical results with standard commercial amboceptors made in the usual way when parallel tests were run.

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A RAPID METHOD FOR DRYING OXALATE SOLUTIONS

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THE preparation of oxalate tubes for collecting and preventing the clotting of blood is in many laboratories and offices a problem of great concern. Several books on clinical procedures recommend that tubes containing the oxalate solution be laid flat on a hot plate, rotated, and the contents allowed to splatter. Such treatment may cause decomposition of the oxalate preparations. This is especially true of oxalate anticoagulants made with ammonium and potassium oxalate. Directions usually given with such a mixture specify that the solution should be spread in a film over the lower walls and dried in an oven at not over 50° C. In addition to being a long, tedious process, such drying usually results in the formation of large crystals that of course go into solution slowly.

We have for some time been using a technique that we feel is most rapid and yet gives oxalate tubes with a thin film over the entire inner surface. In addition, there is no danger of oxalate breakdown by excessive heat.

The technique reported is extremely simple and requires only equipment available in any laboratory. The method is essentially that of lyophilization. The tube to be dried is stoppered with a one-hole soft rubber stopper which contains a short glass tube of a maximum size compatible with the size of tube used. The glass tube is connected to a good grade water pump by flexible rubber tubing heavy enough to withstand the negative pressure of the pump. Adjacent to such a setup should be a burner or hot plate to maintain a beaker of water at from 80 to 100° C.

DRYING OXALATE SOLUTIONS

Drying of the oxalate is accomplished by connecting the tube to be dried to the vacuum system and putting a maximum negative pressure on the system. The tube may now be held in a horizontal position by the left hand. Repeated and rapid snapping blows to the lower part of the tube with the right hand will distribute the oxalate over the entire inner surface of the tube. The tube should occasionally be dipped in the container of hot water to facilitate the evaporation of the water in the tube.

Test tubes or small flasks or bottles containing as much as from 1 to 1½ c.c. of oxalate solution may be drier in not over 2 or 3 minutes by this technique.

If a mechanical vacuum pump is available and provision is made for trapping the water that is distilled, even more rapid drying may be accomplished.

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BOOK REVIEWS

A. A. A. S. Research Conference on Cancer. Edited by Forest Ray Moulton. Publication of the American Association for the Advancement of Science, Smithsonian Institution Building, Washington, D. C. Science Press Printing Company, Lancaster, Pa., 1945. Price \$450. Cloth with 333 pages.

The first A. A. A. S. monograph on cancer research was published in 1936 and was entitled *Some Fundamental Aspects of the Cancer Problem*. The second volume, *A. A. A. S. Research Conference on Cancer*, like the first volume, records in a measure the status of cancer research in 1944. The conference papers are divided into five groups as follows: on the virus approach, on carcinogenesis, on enzymes, on diets, and on chemotherapy.

The first article is on Virus Infection as an Etiologic Agent of Cancer by F. Duran-Reynals and E. W. Shrigley. This is an interesting paper on the possible role of viruses as causative agents of cancer; the authors compare and discuss the viruses that cause cell destruction or cell proliferation. The similarity of both necrotizing and neoplastic infections induced by viruses are compared with respect to the influence of genes, hormones, and physical and chemical agents on their activity as well as factors associated with age, as in cancer. A most interesting phase of this paper is the section on the viruses in relation to the effects they produce with respect to the age and resistance of the host. The section on the fundamental properties of viruses in relation to cancer in which the latency, variation, and masking of viruses are discussed is of particular interest to those in the cancer field.

The second paper is on the Virus Production of Mammalian Tumors by A. Taylor and A. Kynette. In this paper, the authors describe completely their work on the transmission of mammary tumors in mice from cell-free filtrates attained by use of the yolk sac technique for growing tumor tissue. Both Berkefeld filtrates and dried material from tumor-bearing eggs were capable of producing malignant tumors when injected into mice. However, reproducible procedures had not yet been developed, and the number of results obtained with Berkefeld filtrates was only positive in three experiments out of sixty, and the tumors produced as being of viral origin are open to question according to the following papers: Attempts to Demonstrate a Virus-like Principle in Mammalian Tumors by the Yolk Injection Technique by W. R. Bryan, H. Kahler, and V. T. Riley; Yolk Sac Cultivation and Virus Induction of Malignant Tumors by F. R. Heilmann; and Virus Induction of Mammalian Tumors by G. H. Twombly.

H. Kahler and W. R. Bryan in the next paper, Characterization and Separation of Some Macromolecular Components in Tumors and Normal Tissue, show that the finding of a nucleoprotein (ribose type) is not evidence of a virus since both mouse milk containing the milk factor and normal tissues contain nucleoproteins of the same type.

A complete survey on the Inciting Influences in the Etiology of Mammary Cancer in Mice is given by J. J. Bittner. The three inciting influences (the inherited susceptibility, the hormonal stimulation, and the agent transferred in the mother's milk) which have been recognized as causative factors in the production of spontaneous mammary carcinoma in mice are described in detail.

Another paper on the Properties and Nature of the Milk Agent in the Genesis of Mammary Tumors in Mice is written by M. B. Shimken and H. B. Andervont. In this paper which deals with the biologic data, it is shown that the agent is positive in action and also on some of its physical and chemical properties.

A short note on Nonhereditary Nursing Influence in Leukemia by J. Furth follows.

The next series of papers deal with carcinogenesis, and the first, Hydrocarbon Carcinogenesis by L. F. Fieser to whom cancer research owes much, deals with the correlation between structure and activity of the carcinogenic hydrocarbons. The unusual reactivity of these carcinogens with other chemicals in vitro is discussed, and from these characteristics of

the hydrocarbons several hypotheses are presented as possible explanations for the initiation of cancer by the reactivity of these compounds.

In the Relationship of Pyrrol Compounds to Carcinogenesis, presented by F. H. J. Figge, are discussed three hypothetical factors (Factor I, Carcinogenic Steroidal Hydrocarbons or Other Compounds; Factor II, Metabolizing or Oxidizing Agents; and Factor III, Sensitizing Agents) which interact to produce cancer. The porphyrins hypothetically act as sensitizers to carcinogenic stimuli.

The next article deals with the Time and Site of Origin of the Leukemic Cell by J. Furth and M. C. Boon. This study deals with the time of origin (during young adult life and shortly before the onset of malignancy) and the site, which either may be in numerous foci or in one or a few foci, of the leukemic cell in Ak mice.

A summary of Certain Data on the Production of Malignancy in Vitro by W. R. Earle presents the mode of action of methyleholanthrene on connective tissue fibroblasts grown in vitro in a completely heterologous medium for five years. Characteristics of the untreated and treated fibroblasts with respect to the average increase in the width of the cultures, percentage of tumors produced after inoculation in C3H mice, rate of tumor growth in the latter, anaerobic glycolysis, distally located Golgi material, and percentage of vascular tissue elicited upon transplantation are discussed with relationship to the effect of the carcinogen upon the fibroblasts.

The next article is the Occurrence of Dependent and Autonomous Phases in the Development of Cancer by H. S. N. Greene. In experiments using his well-known technique of transplanting tumors to the anterior chamber of the eye, the author postulates that a cancer is not malignant unless it will grow heterologously in that organ.

A Possible Common Mitochondrial Origin of the Variational and Virus Diseases in Plants and Cancer in Animals is then presented by H. G. Du Buy and M. W. Woods. This interesting and thought-provoking paper deals with the variational diseases of plants in which alterations in the mitochondria or plastids can be followed by metabolic and cytological changes in a specific cytoplasmic particulate. The investigations of these authors suggest that viruses may be derived by gradual alterations in normally occurring cytoplasmic components, the mitochondria. Once the derived mitochondria or plastid is formed, it is self-perpetuating and moreover it contains a ribose nucleoprotein, which is characteristic of animal viruses.

The first paper on enzymes, Biochemical Studies of Chemical Carcinogenesis, With a Preliminary Note on the Susceptibility to Chemicals of Certain Neoplasms, both Animal and Human, by C. J. Kensler and C. P. Rhoads, describes the biochemistry of p-dimethylaminoazobenzene, which produces tumors of the liver in the rat. The results of the fine experiments on the inhibitory effect of the metabolites of p-dimethylaminoazobenzene upon a diphosphopyridine nucleotide from yeast, which ferments glucose, cocarboxylase-carboxylase system, upon the respiration of liver slices, upon the oxygen consumption of liver brei, and upon succinic dehydrogenase and cytochrome oxidase are of great value for an understanding of the process of carcinogenesis. A portion of the paper deals with the correlation of inhibitory effects of the split products of methyl derivatives of aminoazobenzene on enzyme systems and cellular respiration with the carcinogenic potency of the parent azo molecule.

The contribution by J. P. Greenstein on Enzymes in Normal and Neoplastic Animal Tissues is a composite picture of the authors excellent investigations on enzyme patterns in normal and neoplastic tissues. Using a number of enzymatic activities of normal mouse tissues and neoplastic tissues of the mouse, six generalizations are made comparing the enzyme activities of both groups of tissues. The generalizations serve to characterize more exactly the biochemical nature of normal and malignant growths. In a similar manner the author has investigated the enzyme activities of the liver of the rat and of the normal adult, regenerating, fetal, and hepatoma and has found that enzymatic patterns of the hepatoma and liver of the fetal liver resemble each other more than do the similar normal adult and regenerating liver. Normal and neoplastic human tissues are also placed into categories depending upon the response of the tissue suspensions to p-phenylenediamine in the presence of an excess of

An abstract of Distribution of Enzymatic Activities in Fractions in Mammalian Liver by A. Claude describes the distribution of cytoplasmic enzymes associated with the free secretory granules, the mitochondria, the microsome fraction, and the supernato.

The Serology of Cathepsins by M. E. Marer and J. W. Thompson is a study on the cathepsins, which are endocellular proteinases responsible for the synthesis and lysis of tissue proteins, and is obviously of great importance in growth phenomena.

Reducing Properties of Serum From Malignant and Nonmalignant Patients and From Normal Individuals by B. J. Savignac, J. C. Gant, and I. W. Sizer is the next presentation in which the time to reduce methylene blue by the serum of normal, noncancerous and cancerous patients is discussed. The reducing activity was found to be located in the albumin fraction and is due to the liberation of sulfhydryl groups by the action of sodium hydroxide.

In the first paper on diet and cancer, the article B Vitamins and Cancer by R. J. Williams, the author describes the investigations in his laboratory on the content of the B vitamins in human and rat normal tissues and in human and rat cancers.

H. P. Rusch, C. A. Baumann, J. A. Miller, and B. E. Kline then describe their studies on Experimental Liver Tumors. The production of tumors on purified diets and the effect of riboflavin and casein, biotin, pyridoxine, and lipids on the genesis of hepatic tumors is described.

A note on Some Aspects of Diet and Cancer by A. Tannebaum follows in which the author stresses the importance of diet in experimental investigations in cancer of mice. His contributions to this phase of cancer research should be carefully read by all in this field of endeavor.

The Effects of Therapeutic Agents on Human and Mouse Leukemia by C. M. Flory is the first of five papers on Chemotherapy of Cancer. In the first paper the effect of many compounds on mouse and human leukemia were tested, and the agents were at best either only palliative or ineffective.

The next article is on the Effect of Various Agents on Normal and Malignant Tissues by H. O. Singher, C. J. Kensler, and C. P. Rhoads. The Warburg method and the egg technique are used to test the effectiveness of various compounds upon normal and malignant tissues.

The results by G. O. Gey, M. K. Gey, F. Inui, and H. Vedder on Penicillin Action on Strains of Normal and Cancer Cells demonstrate no evidence of an increased tumor cell susceptibility by sodium penicillin as compared to normal cells from the results obtained on continuous cultures and on primary explants of tumors produced by inoculations of continuous cultures of tumor cells. The final notes also deal with the influence of penicillin on tumor cultures or in tumor tissue.

CHRISTOPHER CARRUTHERS, M.D.

A NUTRITIONAL SURVEY OF STARVATION IN A GROUP OF YOUNG MEN

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AN OPPORTUNITY was afforded us during July and August, 1945, to observe starvation in a group of German prisoners in a section of Upper Austria. Because of the necessarily short time and the availability of only the simplest of field laboratory methods, the studies were limited. Nevertheless, during the period of starvation of these men, the amount of food eaten was known with considerable accuracy, so that interpretation of the clinical findings can be related to a fairly definite food intake.

The men were seen between two and three months after imprisonment, and during this interval the caloric value of the food eaten varied between 650 and 850 calories daily (see Dietary history under Results). Food eaten before the time of capture is not known and may not have been entirely adequate, so that before the beginning of the period of starvation the nutritional status of these men may not have been optimum.

MATERIALS AND METHODS

One hundred and seventy-one men, appearing to be the most severely undernourished of a larger group, were chosen for the examination. Most of the men had been occupied as heavy workers until they were unable to carry on because of lack of strength. Roughly 10 per cent of those studied were hospitalized because of malnutrition. There was no evidence of tuberculosis or of any acute infectious disease in any of the men included in this group.

The men ranged in age from 16 to 61; the average age was 31 (Table I). Fifty-five per cent were under 30 years of age, and only 6 per cent were over 50.

The method of conducting the survey was a modification of that developed for civilians by the Nutrition Division of the Office of the Surgeon General. The food issue was obtained, a brief physical examination was made on each individual, and blood hemoglobin and serum proteins were determined by the specific gravity method of Lowry and Hastings.¹ Apparatus and materials for these determinations were contained in the field laboratory kit developed by the Army's Medical Nutrition Laboratory. Age, height (in centimeters), and weight (in kilograms) were recorded and compared with standards for the United

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TABLE I. BODY WEIGHT REDUCTION BELOW STANDARD

	NUMBER	KG. BELOW STANDARD	% MORE THAN 10 KG. BELOW STANDARD
All	171	13	70
With edema	41	11	51
No edema	130	13	74
16 to 20 years	62	11	58
20 to 29 years	32	11	56
30 to 49 years	66	16	82
50 years +	11	15	82
Augsburg	994	6	18
Vienna	1,315	9	48

States.² The mean of the deviations from the American standards of all the individuals was determined. Two additional calculations also were made: first, the percentage of all the individuals who were more than 10 per cent below standard in weight; second, the percentage more than 10 kg. below standard in weight.

RESULTS

A. DIETARY HISTORY.—It was possible to calculate with a fair degree of accuracy the food intake of the 171 men during the two to three months of starvation, since the quantities of food issued were known, and it was impossible for the men to supplement the ration. We believe that the distribution was as even as it could be made, although at times an attempt was made to give the workers and those hospitalized for malnutrition more than their equal share of the food available. A sample menu is given in Table II. The calculation of the diet is only approximate, for the varieties of bread, meat, flour, cheese, and fresh vegetables changed from time to time. The caloric value of the food eaten varied between 650 and 850 calories daily during the two to three months considered here.

It may readily be seen that the diet was grossly deficient in calories, chiefly from the extremely low fat and carbohydrate content. Protein was low, but it may have been of high enough biologic value and sufficient in quantity to maintain nitrogen balance had the caloric value of the food been high enough. The calculated vitamin and mineral intake in the diet is certainly very low, and the former is probably higher than that in the food eaten, since most of the food-stuffs were cooked together in the form of a soup.

B. NUTRITIONAL STATUS.—

1. *General Appearance.*—Starvation was evident on inspection (Fig. 1). Most striking was the extreme loss of flesh, especially of body fat (Fig. 2). The usual deposits of body fat were gone, skin was loose, and it showed an almost complete absence of subcutaneous tissue. Posture was generally poor with stooped shoulders and kyphosis evident (Fig. 3). Weakness was obvious in many, and actual syncope occurred in several while waiting for physical examination. A characteristic lethargy was apparent in all of the men, being very severe in those appearing most malnourished. Facial expressions were dull and rarely changed.

TABLE 11. APPROXIMATE COMPOSITION OF FOOD ISSUED DAILY

	GM.	CALORIES	PROTEIN (GM.)	CARBO- HYDRATE (GM.)	FAT (GM.)	CALCIUM (MG.)	IRON (MG.)	SODIUM CHLORIDE (GM.)	VITAMIN A (I. U.)	VITAMIN B ₁ (MG.)	VITAMIN B ₂ (MG.)	NIACIN (MG.)	VITAMIN C (MG.)
Bread, black	100	210	10	49	1	21	2.3	1.7	0	0.2	0	1.3	0
Meat	50	70	10	1	3	7	1.0	0	26	0.04	0.1	1.3	0
Flour	30	104	3	23	0	9	1.5	0.04	7	0.1	0.05	0.9	0
Butter	10	81	0	0	0	2	0	0.2	425	0	0	0	0
Dried legumes	55	193	11	32	1	46	3.1	0.03	111	0.2	0.15	1.5	10
Fresh vegetables	50	S.	1	1	0	39	2.0	0.06	5,000	0.08	0.14	0.4	0
Cheese	10	35	2	0	3	68	0	7	375	0	0.2	0.2	0
Sugar	15	60	0	15	0	0	0	0	0	0	0	0	0
Total		791	40	121	17	195	9.9	2.03	6,247	0.62	0.61	8.6	10

2. Complaints and Symptoms.—

(a) Weakness and easy fatigability were spontaneous complaints in almost all of the men. They found that simple tasks such as arising from bed and walking a short distance required much effort, were difficult to accomplish, and produced undue fatigue requiring long periods for recovery. Most of the

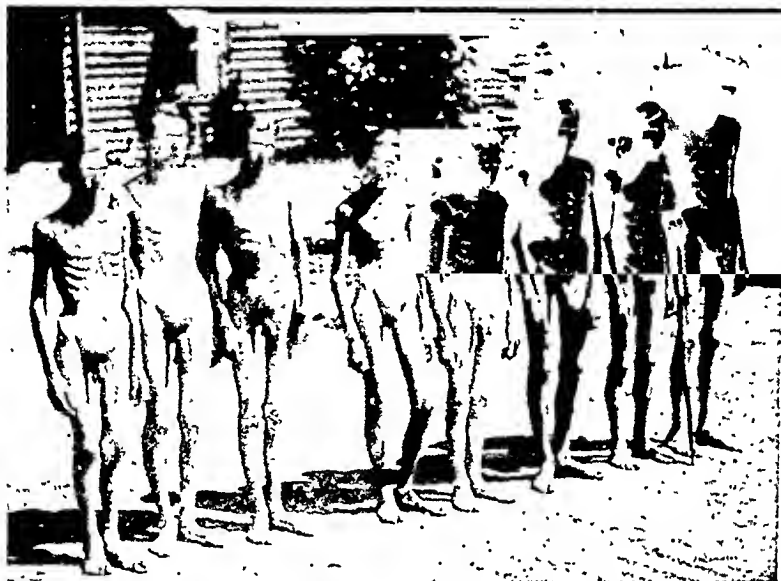


Fig. 1.



Fig. 2.

men remained in bed or were otherwise without activity most of the time. Vertigo and syncope were also common complaints, especially noticeable on arising from the horizontal position or when required to stand for even short periods of time.

(b) Pain in the hips, knees, and ankles, most prominently in the knees, was an extremely common complaint but bore no relation to the incidence of edema. The men observed this when walking, and often a prolonged period of rest was required for improvement. The pain appeared to be in the joints themselves. No joint abnormalities were observed on physical examination.

(c) Diarrhea was complained of by a number of men and was thought to be noninfectious in nature, but unfortunately no stool cultures could be done. Of the men with this complaint, many had several attacks, lasting for a day or so, each with free intervals of as much as two weeks.

(d) Paresthesias of the hands and feet were frequent complaints. This symptom will be discussed under Cardiovascular and Related Phenomena.



Fig. 3.

3. Physical Examination.—

(a) *Measurements:* Reduction in weight compared to United States standards² was present in all instances, the mean reduction being 13 kg. (Table I). Furthermore, 70 per cent of the men were more than 10 kg. below the standard. As might be expected, the younger age groups showed the least reduction, while of those over 30 years of age, 82 per cent were more than 10 kg. below the standard. Weight changes similarly obtained in the cities of Augsburg and Vienna are included for comparison. In Fig. 4 is shown the distribution of the percentage of body weight below the standard. It may be seen that 40 per cent of the group were more than 20 per cent below the standard; that is, for a 180-pound man a reduction of thirty-six pounds or more.

(b) *Integument:*

(1) *Pigmentation.* A variable amount of pigmentation of the skin was seen in many of the men. The pigmentation was most marked on the

exposed areas of the body, and most of the men had fair skin which tans easily. Nevertheless, certain characteristics of the pigmentation suggested that it was not entirely due to exposure. It was deeper in color and often appeared almost black. A few men seen elsewhere recovering from starvation showed a desquamation of pigmented skin, leaving a clear nonpigmented skin in its place. The pigmentation was not accentuated in the skin folds and creases, nor was it observed on the mucous membranes of the buccal cavity.

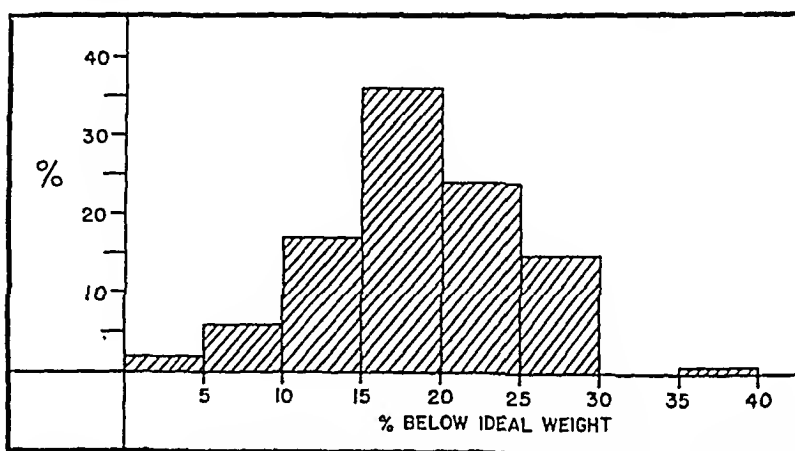


Fig. 4—Distribution of weight reduction below standard expressed as percentage of standard or ideal.

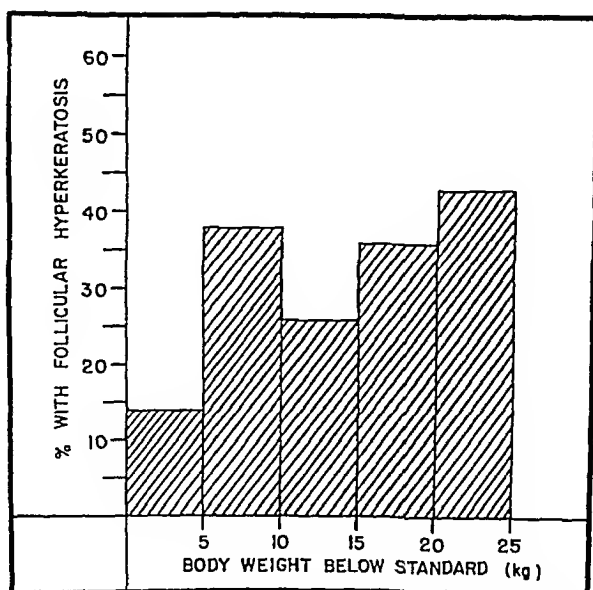


Fig. 5—Incidence of follicular hyperkeratosis as related to reduction of body weight below standard

(2) Follicular Hyperkeratosis. Fifty-four patients (32 per cent of the 171 men) were observed to have skin lesions similar to those described by Youmans and Corlette² and others and thought to be related to vitamin A deficiency. The lesions were usually present either on the extensor surfaces of the upper arms and frequently spread onto the shoulders or on the extensor surfaces of the thighs and buttocks, or, in extreme cases, both the upper and lower extremities were involved. There was found to be a rough parallelism between reduction in weight below the standard and the incidence of follicular hyperkeratosis (Fig. 5). Thus, of those patients who were 5 kg. or less below standard, only 14 per cent showed skin changes compared to 43 per cent for those from 21 to 25 kg. below standard.

(3) Sweat glands. A phenomenon occasionally seen in well-nourished individuals was observed in the men with pigmentation. Vesicles measuring from 1 to 5 mm. in diameter were scattered over the shoulders and upper trunk. They were easily broken and contained clear waterlike fluid. There was no reaction surrounding these lesions, which appeared to be sweat glands covered with a thin membrane of undesquamated skin. The men with these lesions said that in the sun other lesions appeared soon after one crop was broken.

(4) Other skin manifestations sometimes associated with deficiency disease. No instances of purpura, typical pellagrous dermatitis, or the so-called nasolabial seborrhea were seen. Scurvy was not seen.

(c) *Eye, Mouth, and Tongue Changes:*

(1) No instance of conjunctivitis was observed.

(2) There were four men with bilateral circumcorneal injection together with apparent ingrowth of capillaries into the cornea. These lesions resembled those described as due to riboflavin deficiency.⁵

(3) None of the corneal changes related to vitamin A deficiency were observed.⁴

(4) There was no instance of angular stomatitis and cheilosis as characterized by fissuring at the mouth corners and redness and denudation of the lips.

(5) There were only three men with change in the color of the tongue. In two, it was beefy red in color and in one, purple. None of these three showed papillary atrophy, but eight men did have moderate atrophy of the papillae of the tongue. None of these men was edentulous.

(6) Gingivitis was observed in a number of men, but in each instance it was associated with marked dental caries so that it could not be definitely associated with vitamin C deficiency. As already stated, there was no scurvy.

(d) *Cardiovascular and Related Phenomena:* Moderate hypotension and marked bradycardia were the rule (Fig. 6). The pulse rate and blood pressure were taken in sitting position but not at rest, as the determinations formed part of an active examination. Thirteen per cent of the men had pulse rates between 20 and 40 per minute; 71 per cent, between 41 and 60; 13 per cent, between 61 and 80; and only 3 per cent, above 80. There was no definite correlation between pulse rate and reduction in body weight below the standards.

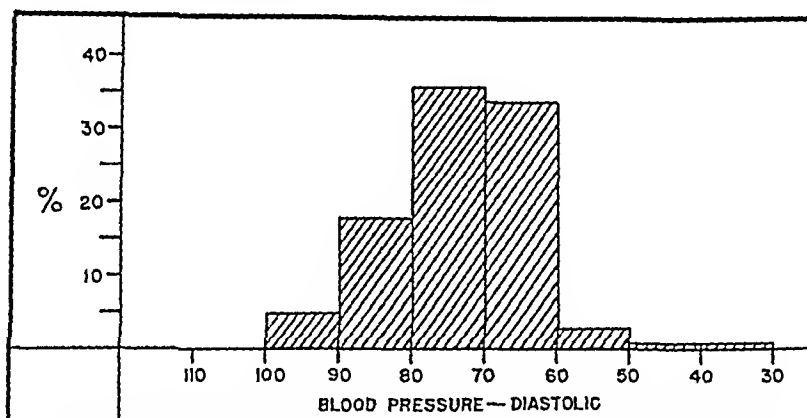
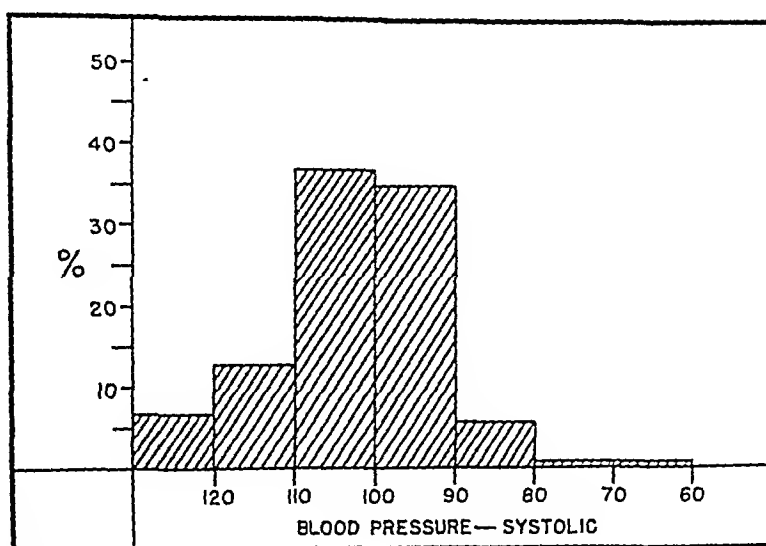


Fig. 6.—Blood pressure related to weight reduction below standards.

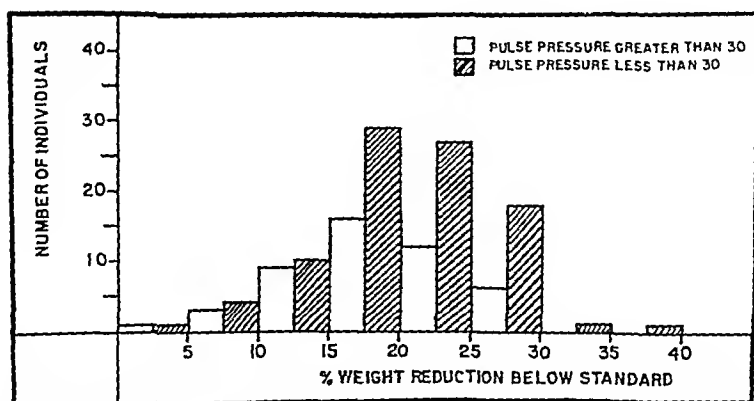


Fig. 7.—Relation between pulse pressure and percentage reduction in weight below standard.



Fig. 8.

The blood pressure decrease was most marked in the systolic rather than the diastolic pressure, producing a diminished pulse pressure. In general, the pulse pressure was least in those men whose body weight was the greatest below the standard (Fig. 7).

Measurements of basal oxygen consumption were not possible. Realizing the inherent inaccuracies, calculation of the metabolic rate from Read's formula was done in thirty-two patients who, of course, were not under basal conditions. The metabolic rate so calculated ranged from -10 to -55 per cent, with an average of -28 per cent.

A phenomenon resembling acrocyanosis was observed in the skin of the hands up to the lower forearm (Fig. 8) and in the feet to just above the ankle. On a cool morning, these areas, particularly the hands, were cold to the touch and presented a mottled cyanotic appearance. During the warmer part of the day, the cyanosis sometimes gave way to a mottled reddish flushing, but the hands were still unusually cold to the touch. In a few instances there was a brownish pigmentation and occasionally a fine desquamation of the skin over the same areas.

This phenomenon resembling acrocyanosis was observed in 75 per cent of the men and generally was most marked in those patients with the greatest reduction in weight below the standard.

Sixty-five per cent of the men with the acrocyanotic appearance of the hands and feet complained of paresthesias consisting of numbness, tingling, or a burning sensation, most marked in the fingertips and toes but often extending into the hands and feet and even occasionally to the forearms and lower legs. Paresthesias of this variety were present in 24 per cent of the men examined. The acrocyanotic phenomenon suggested that the paresthesias were largely on a vascular basis rather than associated with peripheral neuritis or cord changes.

(e) *Extremities:*

(1) Nerve lesions. Calf muscle tenderness was observed in only three instances. There were two individuals with possible peripheral nerve palsy, one with peroneal palsy, unilateral, and the other with absent knee jerks. There were no other signs of thiamine deficiency in these two men. In all others the knee jerks and vibratory sense tested at the base of the great toes were present and apparently normal.

(2) Strength of grip. The ergometer was used to test the strength of the men in twenty-four instances. Each was given three trials; almost without exception, the first trial was the best, the second was much weaker, and on the third trial many of the men could barely move the needle. The best reading of the three was taken in each case and was essentially normal for the machine used. Significant only was the marked reduction on the second and third trials.

(3) Leg ulcerations. A number of the men had one or more leg ulcers, usually on the lower third of the leg. They were generally clean, but with little evidence of granulation, and had a punched-out appearance with sharp edges. The men said that these lesions were very slow in healing.

(f) *Edema and Serum Proteins:* There were forty-one men with varying degrees of dependent edema (Fig. 9). Many others who had been in bed

stated that they had swelling of the feet and ankles when they were up for an hour or so and that it as rapidly vanished when they reclined again. No sacral edema was seen.

The edema was of a soft, easily pitting character, always seen in the feet and ankles and sometimes as far up as the thighs. Only one man was seen with edema of the anterior abdominal wall. Several had evidence of ascites, as well as advanced leg edema. Edema of the face was occasionally seen and was most obvious in the eyelids, although many men volunteered that their face and eyes were swollen on arising in the morning.



Fig. 9.

In general, the men with edema were of middle age, the young and the old being spared to a large extent. The mean reduction below the standard for body weight in persons with edema was 11 kg. (17 per cent) in contrast to 13 kg. for those without edema (Table I). Two factors appeared to account for the smaller reduction in weight. First, the body weight included a variable amount of edema fluid. Second, the men with edema often appeared to be better nourished than those without and were more likely to be of the stocky muscular type of build. In addition, there were many men with evidence of extreme weight loss but without any evidence or history of edema.

Fifty-one total serum protein determinations were made, eighteen of them on men with edema, with an average of 5.7 Gm. per 100 c.c. and with the lowest of 4.0 Gm. per 100 c.c. Thirty-six per cent of the serum proteins determined were 5.5 Gm. per 100 c.c. or below. Of the men without edema only 29 per cent were 5.5 Gm. per 100 c.c. or below, while of those with edema 47 per cent were in this low range (Fig. 10). Thus, whereas there were many men with

edema and normal proteins and men with low proteins and no edema, there was a definitely higher percentage of hypoproteinemia in those with edema. There was no correlation between weight reduction below the standard and serum protein concentration.

Signs which might be interpreted as indicating a deficiency of one or more of the members of the vitamin B complex were very few and were generally distributed evenly among those with and without edema. Most men with a

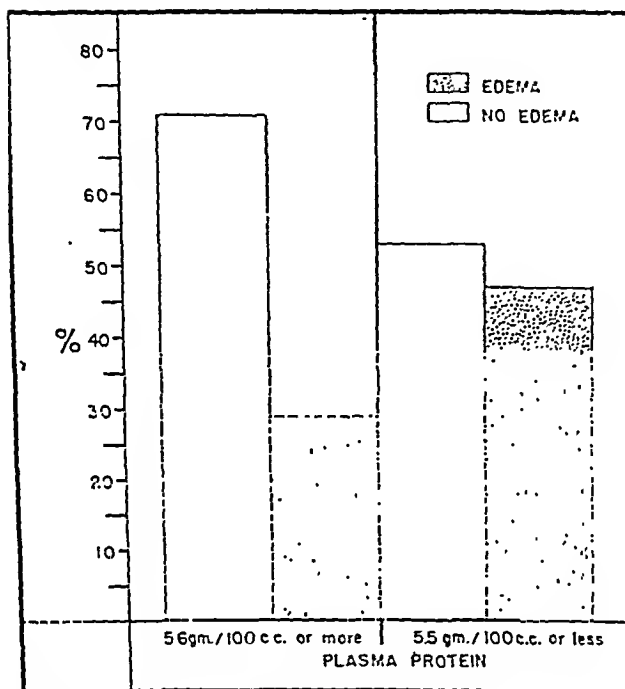


Fig. 10.—Incidence of edema in relation to serum protein level.

significant amount of edema complained of pain in the feet and ankles, and two of the three men with calf muscle tenderness had edema, suggesting a possible thiamine deficiency in these two men. Other than this possibility, there seemed to be no evidence pointing to vitamin deficiency as a cause for edema.

(g) *Anemia*: Sixty blood hemoglobin determinations and eighteen red blood cell counts were made. Only two men had hemoglobin concentrations below 10 Gm. per 100 c.c., or 64 per cent. Red cell counts were not done on these men. Eight of the eighteen red cell counts were below 4 million per cubic millimeter, but only one was below 3 million (2.9 million). This man had a hemoglobin concentration of 79 per cent and thus a color index of 1.4. This is the only instance of macrocytosis observed. The remainder of the color indices were from 0.8 to 1.1. Thus, it can be said that anemia was uncommon and when present was more likely to be normocytic and normochromic than hypochromic. Hemoglobin concentrations were no lower in those with edema than in those without. There was no definite correlation between reduction of body weight below standard and the hemoglobin concentration.

COMMENT

Starvation and malnutrition may be exhibited in quite a variety of ways in different individuals, depending on body stores of nutrients, environmental circumstances, and what foods are available for consumption. Starvation is primarily a deficiency of calories which results in a protein deficiency, because the body uses protein for caloric purposes. Vitamin deficiencies, particularly those of the water-soluble vitamins, may not be expected to be frequent in starvation because of the general decrease in metabolism. The men observed in this study had food intakes which showed evidence of deficiency almost exclusively of calories and protein.

Certain of the observations require special comment.

1. Pigmentation. It may be that the general skin pigmentation and related skin changes described were related only to exposure, but the distribution, intensity, and tendency to desquamation during recovery lead one to believe that other factors were concerned. There was no resemblance to the pigmentation of adrenal insufficiency or to pellagrous dermatitis. It is our opinion that the skin changes represent a slowing down of the usual skin replacement and desquamation, just as other body tissues are living at a slowed pace. Thus accumulation of skin rather than normal production and desquamation led to piling up of skin exposed to sun and the elements. The same explanation may account for the appearance in some of the men of vesicles representing sweat glands covered by undesquamated skin. The occurrence in these men of small, poorly healing leg ulcers may also represent a slowly reproducing skin rather than a specific vitamin deficiency. The low intake of vitamin C in these men, however, may have influenced the pigmentation,⁶ the skin changes, and also the poor healing of the leg ulcers.⁷

2. Follicular hyperkeratosis. The incidence of this sign was certainly high in these men. The diet was very low in vitamin A, although it was fairly adequate considering carotene as provitamin A. The extremely low fat content of the diet undoubtedly impaired absorption of carotene and may have hindered conversion of carotene to vitamin A. Similar findings are mentioned in other reports on starvation. How much the lesions were related to a deficiency of this vitamin, and how much to lowered skin productivity as previously suggested, cannot be stated categorically. It is noteworthy that none of the eye signs described for vitamin A deficiency were observed.

3. Lesions resembling acrocyanosis. The lesions of the hands and feet resembling acrocyanosis and often associated with paresthesias were at once striking and difficult to explain. Similar changes of a milder degree are occasionally seen in thin adolescents. Individuals with peripheral neuritis as a rule have unstable neurovascular mechanisms in the affected parts, but there was very little confirmatory evidence of peripheral neuritis in these men.

Paresthesias and possible vascular changes in the hands have been ascribed to poor posture with drooping shoulders and pressure on the brachial plexus.⁸ This may in part explain the phenomena observed in these men who certainly exhibited poor posture. Butler and co-workers⁹ reported paresthesias in the

group of internees they studied, but they did not relate it to any specific deficiency. The high incidence of loss of vibratory sense found by these authors was not present in the group of men reported here nor in studies on starvation in Holland.¹⁰

4. The occurrence of "hunger edema" in 24 per cent of the men appears at first thought to be evidence of marked protein deficiency with hypoproteinemia. This was in part true; that is, there was a higher incidence of edema in those with hypoproteinemia than in those without (Fig. 8). Nevertheless, there were a significant number of men with edema but relatively normal serum protein concentrations. This lack of correlation between edema and the serum proteins in starvation has been observed by others.^{10, 11} Several possible explanations appear for this. First, no partition between the albumin-globulin fractions was possible in this study, so that a hypoalbuminemia may have been masked by a hyperglobulinemia. Second, the edema in some of the men may have been associated with a thiamine deficiency. However, lack of other evidence of such a deficiency, the amount of thiamine in the diet, and the absence of signs of beriberi heart disease make this explanation unlikely. Reduction in blood volume in starvation also has been reported.¹⁰ It has been suggested that this may mask a reduction in the total circulating serum proteins, but it is difficult to see how this could be related to edema formation since the plasma osmotic pressure is related to the concentration rather than to the total amount of serum protein. Furthermore, Keys, in experimental starvation in man,¹¹ reports only a small decrease in total blood volume made up of a proportionately greater reduction in volume of cells and an actual, although small, increase in plasma volume. At present the discrepancies between the occurrence of edema and the serum protein level need further clarification.

5. Anemia in these men was definite although not marked. In general it was normocytic and normochromic. As with the serum proteins, it may be thought that reduced blood volume masks a more severe anemia. However, the findings reported by Keys,¹¹ referred to previously, show a relatively greater reduction in red cell volume than in total blood volume.

6. The calculated (therefore, probably maximum) intake of these men of thiamine, riboflavin, niacin, and ascorbic acid, during the two or three months of starvation, is certainly low. The small incidence of signs classically related to deficiency of the water-soluble vitamins (B complex and C) suggests that the intake, although small, was sufficient during this time and with reduced calorie intake and general body metabolism to prevent specific deficiencies. For thiamine, Holt¹² suggests that between 0.24 and 0.44 mg. per 1,000 calories is protective. The requirements for riboflavin and niacin are less well known, but the intake in these men at this calorie level and for the time of starvation was sufficient to prevent gross signs suggestive of deficiencies of these vitamins. With respect to ascorbic acid, it is known that at least two months is required for the appearance of scurvy on a scorbutic diet. Thus, Crandon, Lund, and Dill,¹³ in experimental human scurvy, found that over four months were required before the lesions appeared.

SUMMARY

1. A nutritional survey of 171 men suffering from a severe degree of starvation is reported.
2. The diet of these men averaged about 750 calories per man per day for from two to three months before the study.
3. Extreme wasting was evident upon inspection and was usually associated with lethargy, a stooped posture, weakness, and often actual syncope. Comparison of actual weight with data given in standard height and weight tables showed that all the men were under the standard in weight, the average being 13 kg. below the standard. Seventy per cent were more than 10 kg. below the standard.
4. Joint pains, prominently in the knees, were common.
5. Diarrhea of a recurrent variety was frequently a complaint.
6. A generalized pigmentation was observed.
7. Clinical evidence suggesting a deficiency of any of the vitamins was lacking except for the following: a high incidence of follicular hyperkeratosis, four instances of circumcorneal injection, three instances of color change in the tongue, and eight instances of papillary atrophy of the tongue. Eye signs suggesting vitamin A deficiency were not observed.
8. A condition resembling acrocyanosis was observed in the distal extremities of many of these men and was often associated with paresthesias.
9. Edema, probably of nutritional origin, was observed in 24 per cent of the men. Hypoproteinemia was common but was not present in a significant number of the men with edema.
10. Anemia was not severe, only two men having a hemoglobin concentration below 10 Gm. per 100 c.c. of blood.

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THE ACTION OF THIOBISMOL* ON THERAPEUTIC QUARTAN MALARIA

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SCHWARTZ,¹ in 1939, first introduced sodium bismuth thioglycollate (thio-bismol) as an effective agent for reducing the frequency of vivax malaria paroxysms without completely eliminating them. Other workers² subsequently confirmed the value of this drug in therapeutic malaria. Prior to this investigative work a variety of agents, including small amounts of quinine, arsenicals, and intravenous 20 per cent dextrose solution, was used unsuccessfully for regulating or temporarily interrupting malaria paroxysms.

Young, McLendon, and Smarr³ clearly demonstrated that sodium bismuth thioglycollate in 0.1 or 0.2 Gm. amounts had an inhibitory effect against half-grown *Plasmodium vivax* parasites. Older or younger parasites were not affected in their series. Paroxysms, which were converted from quotidian to tertian periodicity, usually remained tertian for the duration of the infection and often through several subinoculations. The use of this drug, therefore, in the treatment of neurosyphilis with therapeutic malaria, allowed a full course of therapy without rapidly exhausting the patient from daily febrile elevations. It also facilitated temporary interruptions of malaria when these were indicated by minor complications.

Sodium bismuth thioglycollate was first used in quartan malaria by Young, McLendon, and Smarr,³ in 1943, but no consistent results were obtained. Thirty-seven injections were administered to nine Negro patients showing either one, two, or three broods of *Plasmodium malariae* parasites. Occasional, unpredictable interruptions occurred, but no correlation of the patients' previous cycles, the time of administration of the drug, the clinical effect, or the effect on the parasitemia was offered.

Attention was directed first to the action of thiobismol on quartan malaria at this Neurosyphilis Center, when an attempt to interrupt temporarily the vivax parasitemia in a patient experiencing a mixed *P. vivax* and *P. malariae* infection resulted in the drug's unexpected elimination of the quartan parasitemia with little effect on the vivax cycle. In retrospect, it was evident that, at the time of injection, the vivax parasites were mature and, therefore, according to previous studies, not susceptible. The action of sodium bismuth thioglycollate on quartan malaria, however, was further investigated. Two and three daily injections, respectively, were given to two patients undergoing quartan malaria therapy. Both experienced prolonged interruptions, with afebrile periods lasting for more than sixteen days. Since this prolonged

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*Thiobismol, sodium bismuth thioglycollate.

effect was not particularly desirable, only single injections of the drug were used in the remainder of the study.

The purpose of this investigation was twofold: (1) to determine whether sodium bismuth thioglycollate inhibits half-grown *P. malariae* trophozoites, an action similar to that observed in vivax malaria, and (2) to study the regulating effect of the drug in irregular quartan malaria cycles, noting especially the optimal time of administration for the predictable interruption of individual paroxysms.

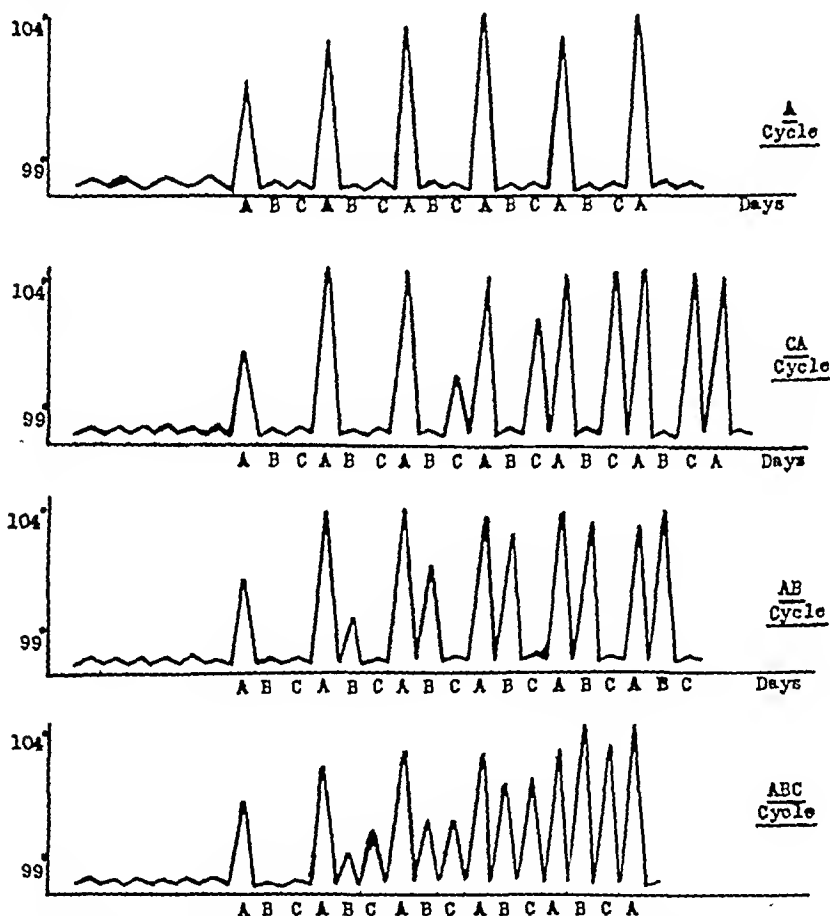


Fig. 1.—Examples of temperature graphs observed with the various quartan malaria cycles.

MATERIAL AND METHOD

Fifty-six injections of sodium bismuth thioglycollate were administered to thirty-eight patients (six white, thirty-two Negro) in this series. Three of the thiobismol injections were given in doses of 0.1 Gm. and the remaining fifty-three in doses of 0.2 Gm. Quartan malaria was induced routinely by blood inoculation intravenously with varying parasite dosages. The quartan cycles encountered varied from the true quartan cycle with one paroxysm occurring every third day to the quotidian quartan cycle with a paroxysm occurring daily. The thiobismol used* was injected intramuscularly in all instances. No

*A preparation of Parke, Davis & Company, Detroit, Mich.

arbitrary schedule of injections was followed, for it was thought that chance distribution over the period of seventy-two hours from the onset of the main quartan paroxysm would reveal more pertinent information.

Quartan malaria usually produces a single paroxysm every third day, two daily paroxysms alternating with one fever-free day, or three daily paroxysms with no fever-free day. Occasionally, more irregular courses with poor delineation of paroxysms may occur. The main cycles have arbitrarily been designated by most investigators as true quartan (one paroxysm every three days), double quartan (two paroxysms every three days), and quotidian quartan (three paroxysms every three days). Those paroxysms appearing in a true quartan cycle are usually referred to as the *A* paroxysms or cycle. Parasites causing the *A* cycle, that is, maturing during the *A* paroxysms, were designated the *A* brood. Subsequent intermediate paroxysms on the second or third day following the main cycle have been termed the *B* and *C* paroxysms, respectively. These different quartan cycles with their *A*, *B*, and *C* designations are illustrated graphically in Fig. 1. Quartan malaria, therefore, ordinarily presents one of four types of cycles: *A* (true quartan), *AB* (double quartan), *CA* (double quartan), and *ABC* (quotidian quartan). The *BC* type of cycle, in which the main *A* paroxysms disappear later in the febrile period, is rarely seen and was not encountered in this series. These cycle descriptions have been employed frequently in this paper, since they simplify the subject matter and often indicate the effects of thioibismol more precisely.

TABLE I. CLINICAL EFFECTS OF SODIUM BISMUTH THIOLYCOLLATE ON QUARTAN MALARIA RELATED TIME OF ADMINISTRATION, AGE OF PARASITE BROODS, AND PREVIOUS CYCLES

TIME* OF ADMIN- ISTRA- TION	AGE OF BROODS†			TRUE QUARTAN A				DOUBLE QUARTAN AB				QUOTIDIAN QUARTAN ABC				TOTAL		
				A REMOVED	INTERRUPTION‡	A UNAFFECTED	NO EFFECT	TOTAL	A REMOVED	B REMOVED	TOTAL	A REMOVED	PROLONGED INTERRUPTION§	TOTAL	A REMOVED		B REMOVED	B AND C REMOVED
	A	B	C															
≤6	Mature	$\frac{2}{3}$	$\frac{1}{3}$	-	5	6	11	-	4	4	-	-	-	-	-	1	1	1
7 to 18	$\frac{1}{6}$	$\frac{2}{3}$	$\frac{1}{3}$	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-
19 to 30	$\frac{1}{6}$	Mature	$\frac{2}{3}$	12	6	8	16	1	-	1	-	3	3	-	-	-	2	2
31 to 42	$\frac{1}{6}$	$\frac{1}{6}$	$\frac{2}{3}$	2	-	2	4	-	-	-	-	-	-	1	-	-	1	1
43 to 54	$\frac{2}{3}$	$\frac{1}{3}$	Mature	1	-	5	6	-	-	-	2	-	2	1	-	-	1	1
55 to 66	$\frac{2}{3}$	$\frac{1}{6}$	$\frac{1}{6}$	-	-	2	2	-	-	-	-	-	-	-	1	-	1	1
Total				5	12	23	40	1	4	5	2	3	5	2	1	1	2	51

*Calculated as the number of hours from the onset of the main *A* paroxysm.

†Listed as fractions of maturity, that is, $\frac{1}{2}$ equals half-grown parasites.

‡Interruption for from four to eight days with reappearance of the *A* cycle.

§Prolonged interruption for from sixteen to thirty days after infection.

In contrast to patients with vivax malaria, in whom, at any particular time, there are two different age groups of parasites undergoing schizogony, patients with quartan malaria present three different stages of parasites at one time. Therefore, to determine the regulating effect of sodium bismuth thioglycollate in quartan malaria, the growth stage of the parasite broods causing each of the cycles must be correlated with the time of administration of the drug. This has been done in Table I. For example, twenty-four hours after the onset of the main *A* paroxysm, the *A* parasites are one-third grown, the *B* parasites mature, and the *C* parasites two-thirds grown. The time of administration of thioibismol in this study was calculated as the number of hours from the onset of the main *A* paroxysm.

The following data was tabulated on each patient:

1. Dose of sodium bismuth thioglycollate.

2. Previous cycles (*A*, *AB*, *CA*, or *ABC*) and number of paroxysms experienced prior to injection.

3. Time of administration of sodium bismuth thioglycollate.
4. Effect on parasitemia (drop in parasite count per cubic millimeter listed).
5. Clinical effect, indicating the number of afebrile days following the injection, the subsequent cycle of paroxysms, and the designation of eliminated cycles.
6. The occurrence of an out-of-cycle paroxysm immediately following the injection.

RESULTS AND DISCUSSION

Forty of the fifty-six injections of sodium bismuth thioglycollate were administered to patients experiencing *A* cycles only (Table 1). Definite clinical interruptions occurred in seventeen (42.5 per cent) of these forty patients. Two types of clinical effect were noted. In five patients (12.5 per cent) sodium bismuth thioglycollate caused a temporary interruption lasting from four to eight days, followed by the appearance of a *B* or *C* cycle of paroxysms with the original *A* cycle eliminated. In the remaining twelve patients (30 per cent), of those experiencing definite clinical effects, the temporary interruption of from four to eight days was followed by the reappearance of the *A* cycle with no intermediate paroxysms.

Of eleven injections given to patients at the time when the *A* brood of parasites was mature, five (45.5 per cent) resulted in temporary interruption, but the *A* cycle was unaffected and reappeared. Of four injections given when *A* parasites were half-grown, two (50 per cent) were followed by temporary interruptions with the subsequent appearance of a *B* or *C* cycle and the elimination of the original *A* cycle. In the other patients with definite clinical effects, *A* cycles were also removed when the *A* brood of parasites was one-third and two-thirds grown, and temporary interruption, without affecting the *A* cycles, occurred when they were one-sixth and one-third grown. Although it is obvious that, in patients with true quartan malaria, a definite clinical effect following the injection of sodium bismuth thioglycollate can be expected only irregularly, the parasites inhibited were uniformly partially grown. Little or no effect was observed on cycles produced by mature or very young parasites.

Sixteen injections of sodium bismuth thioglycollate were administered to patients experiencing irregular quartan cycles of the *AB*, *CA*, and *ABC* types. Of these, five were given to patients with the *AB* double quartan cycle. A definite clinical effect followed each of these five injections. In three patients a temporary interruption of from three to six days occurred with a true quartan cycle (*A* or *B* only) supervening. In the remaining two patients there was no sustained interruption of paroxysms, but a single paroxysm was removed, converting the cycle to a true quartan one (*A* or *B*). Four of the five injections were administered when the *A* parasites were mature and the *B* two-thirds grown. The *B* cycle was removed in each of these instances, and the *A* cycle was unaffected. One of the five injections was given when the *B* cycle was mature and the *A* one-third grown, and this resulted in removal of the *A* cycle only. Although injections were given only at these times in the patients with *AB* double quartan malaria, partially grown trophozoites were always inhibited, and no effect was observed on mature parasites.

Five injections were given to patients experiencing the *CA* type of double quartan malaria, and all of these were followed by definite clinical effects. In

three patients prolonged interruption of paroxysms for more than sixteen days occurred. Because of the limitation on the patients' hospitalization and the fact that they had already completed the prescribed course of therapeutic malaria, they were placed on routine atabrine therapy before the return of paroxysms could be noted. In the other two patients temporary interruption of paroxysms for from three to four days ensued, and the subsequent cycle was converted to the true quartan type. These latter two patients received thiobismol when the *A* parasites were two-thirds grown and the *C* parasites mature, and in both instances the *A* paroxysms only were removed. In the three patients experiencing prolonged interruption, the injections were given when the *A* parasites were one-third grown and the *C* parasites two-thirds grown. Thus, the prolonged interruption may have been occasioned by the partial growth of both broods of parasites with simultaneous inhibition by thiobismol. It is probable that paroxysms would have returned had the period of observation been prolonged further.

Of the six injections given to patients experiencing quotidian quartan types of cycle (*ABC*), four were followed by appreciable clinical effects. One, administered when the *A* parasites were mature, the *B* two-thirds grown, and the *C* one-third grown, resulted in the elimination of the *B* and *C* cycles, with conversion to true quartan malaria with the *A* cycle only. Two of the six injections given when the *A* parasites were half-grown and two-thirds grown, respectively, resulted in the elimination of the *A* cycle and conversion of the quotidian course to double quartan malaria of the *BC* type. One other injection, administered when the *B* parasites were half-grown, resulted in the elimination of this cycle and the conversion to a *CA* type of double quartan cycle.

The remaining two of the six injections, given to patients when the *A* parasites were one-third grown, the *B* parasites mature, and the *C* two-thirds grown, resulted in no clinical effect. Although, on the basis of the previous results, inhibition of the partially grown *A* and *C* parasites was expected, the failure may have resulted from the height of the parasitemia in these patients. Immediately prior to the injections of thiobismol, the parasitemia in one patient was recorded as 17,120 per cubic millimeter and in the other as 33,630. These parasite counts were the highest in the entire series of thirty-eight patients. The usual parasite density in quartan malaria encountered in this series ranged from 2,000 to 6,000 parasites per cubic millimeter. In spite of the fact that clinical effects were not observed in these two failures, the parasitemia was markedly affected, with a drop in parasite density within thirty-six hours of the injection to 6,336 per cubic millimeter in one case (40 per cent of the original parasitemia) and to 5,670 in the other case (15 per cent of the original parasitemia). This further evidence would permit one to suppose justifiably that a clinical effect would have resulted had the parasitemia originally been at the usually lower level. Thiobismol, therefore, apparently has a predictable, uniform action in reducing the frequency of paroxysms in double quartan or quotidian quartan malaria cycles, if the parasitemia, prior to injection, is below 10,000 parasites per cubic millimeter.

An analysis of the effect of sodium bismuth thioglycollate on the *P. malariae* parasitemia further supports this conclusion. In Table II is listed the percentage drop in parasite density, correlated with the original height of the parasitemia and the presence or absence of clinical effects following injection. It is noted that none of the patients experiencing definite clinical effects had original parasite counts above 10,000 per cubic millimeter, while five (20 per cent) of the twenty-five injections not followed by clinical effects were given to patients showing high parasitemias ranging from 11,120 to 33,630 per cubic millimeter. These high parasite densities, therefore, although they may be appreciably reduced by the injection of thiobismol, may not reach low enough densities to occasion clinical interruption. It is also interesting to note, however, that two cases showing definite clinical effects were not accompanied by appreciable decreases in parasitemia.

TABLE II. EFFECT OF SODIUM BISMUTH THIOLYCOLLATE ON *P. MALARIAE* PARASITE DENSITIES RELATED TO THE CLINICAL EFFECTS AND ORIGINAL PARASITEMIAS

	ORIGINAL DENSITY†	DEFINITE CLINICAL EFFECT				NO CLINICAL EFFECT				TO- TAL
		0 TO 4	5 TO 10	OVER 10	TO- TAL	0 TO 4	5 TO 10	OVER 10	TO- TAL	
Drop in Parasitemia*	0 to 20	7	4	-	11	-	-	1	1	12
	21 to 40	8	4	-	12	1	2	1	4	16
	41 to 60	1	2	-	3	1	4	2	7	10
	61 to 80	2	1	-	3	1	1	1	3	6
	81 to 100	1	1	-	2	8	2	-	10	12
Total		19	12	0	31	11	9	5	25	56

*Drop in parasitemia calculated as percentage of original parasite density, that is, drop to 5,000 per cubic millimeter from original density of 10,000 per cubic millimeter equals 50 per cent drop in parasitemia.

†Original parasite density in thousands of parasites per cubic millimeter.

In 75 per cent of all instances when sodium bismuth thioglycollate was injected on fever-free days, a paroxysm, usually reaching from 102 to 104° F., was precipitated within from one to four hours following the injection. Such an effect was, of course, masked when the drug was administered during the febrile episode. The previous demonstration that sodium bismuth thioglycollate in quartan malaria as well as in vivax malaria inhibits partially grown trophozoites suggests that the inhibited parasites may prematurely break out of erythrocytes and cause out-of-cycle temperature elevations. At any rate, no correlation between the precipitation of paroxysms and the occurrence of clinical interruption or reduction in frequency of paroxysms was observed.

SUMMARY AND CONCLUSIONS

1. Fifty-six injections of sodium bismuth thioglycollate in doses of 0.1 and 0.2 Gm. were administered at different intervals to thirty-eight patients experiencing true, double, and quotidian types of therapeutic quartan malaria.

2. Only 42.5 per cent of the injections given to patients with true quartan malaria were followed by temporary interruption. However, in all of these cases showing definite clinical effects, only partially (one-third, one-half, or two-thirds) grown parasites were inhibited.

3. Eighty-seven and five-tenths per cent of the injections administered to patients with double quartan or quotidian quartan malaria resulted in temporary interruption and/or reduction in frequency of paroxysms. Partially grown parasites were uniformly affected in these patients, and no mature or very young parasites (less than one-third grown) were influenced by the injections.

4. None of five patients with original parasite densities above 10,000 parasites per cubic millimeter responded to sodium bismuth thioglycollate injections with definite clinical effects. Two of these patients (12.5 per cent of the series of double quartan and quotidian quartan malaria patients) had the highest parasite densities in the entire series. In these instances the injections of thio-bismol were followed by an appreciable decline in parasite count, but no reduction in frequency of paroxysms occurred.

5. Sodium bismuth thioglycollate appears to be an effective drug for reducing the frequency of paroxysms and regulating the febrile cycles in patients experiencing double quartan or quotidian quartan malaria. Its use may enable patients with neurosyphilis to tolerate better complete courses of therapeutic quartan malaria.

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THE DISTRIBUTION OF ATABRINE IN THE BLOOD, THE SKIN, AND ITS APPENDAGES

METHODS FOR THE RAPID AND SIMPLE DETECTION OF THE PRESENCE OF ATABRINE IN BLOOD, SKIN, AND NAILS

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THE importance of an effective and safe antimalarial therapy can hardly be overestimated. Since the introduction of atabrine (Quinaerine hydrochloride, U. S. P. XII) many attempts have been made to make this drug more effective by establishing quickly an effective plasma level for a sufficiently long period of time, for we know that its plasmodioidal effect in vitro is directly dependent upon its concentration.

This undertaking, however, is dependent upon a reliable method to determine atabrine levels in the blood readily. This would enable one to evaluate clinically the feasibility of at least a temporary cure of this disease by establishing a safe and effective atabrine blood level.

The methods used so far have been rather complicated. The older methods¹⁻³ are not sensitive enough to permit control of the plasma level during suppressive treatment and even not during therapeutic attempts. While the studies described in this paper were in progress, Brodie and Udenfriend⁴ published a method which is both sensitive and highly accurate. It is, however, a rather complicated and detailed procedure requiring the skill of a chemist and a well-equipped laboratory. At the same time Masen⁵ published a method which is less complicated, although less sensitive, but still is rather intricate and requires considerable laboratory facilities. After our extraction method was completed, but unpublished due to the general ban on publications concerning malarial therapeutics, a report by Auerbach and Eckert⁶ appeared which described a somewhat similar but improved method.

Since it is well known that atabrine may cause an intense yellow staining of the skin if taken over long periods of time in suppressive doses or in some individuals after one course of therapeutic application, it was interesting to see whether the level of atabrine in the blood was in any way related to the level in the skin which can be determined also by photofluorometric means. The Dermofluorometer developed by one of us (K. L.)⁷ for the purpose of determining the fluorescein content in the skin after an intravenous injection of this dye can be used also with slight changes to determine the content of atabrine in the skin as long as it is fluorescent. This skin fluorescence also may be observed visually by the use of a long-wave ultraviolet light source, thus enabling the observer to judge the distribution of atabrine in the skin and its appendices.

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Aided by a grant from the John and Mary R. Markle Foundation.

A paper with essentially the same content was submitted for publication in the early part of 1944 but was not published on account of the ban on publications on malarial therapeutics then in existence.

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It was the aim of this investigation to find a method for plasma level as well as skin level determinations which could be employed at outlying stations by an average laboratory assistant without the need of extensive laboratory facilities. At the same time it should be accurate enough that by the use of a more sensitive measuring instrument it would be of sufficient accuracy for laboratories with more elaborate facilities.

PROCEDURE

From 15 to 20 c.c. of venous blood are drawn and transferred into a glass-stoppered bottle which contains 2 drops of heparin solution. Plasma and red cells are separated by centrifugation in a graduated test tube to determine the hematocrit. Seven cubic centimeters of plasma are then pipetted into a glass-stoppered, no-lub separatory funnel of 60 c.c. capacity. Two cubic centimeters of 4 N sodium hydroxide solution and 7 c.c. of absolute ether (C.P.) are added. If the fluorophotometer is used, 14 c.c. of ether are added resulting in a ratio of 1:2. The separatory funnel is stoppered and shaken vigorously for four minutes. After standing ten minutes, the aqueous layer is let off and discarded. The supernatant liquid is transferred into an appropriate cuvette which is stoppered to avoid evaporation.

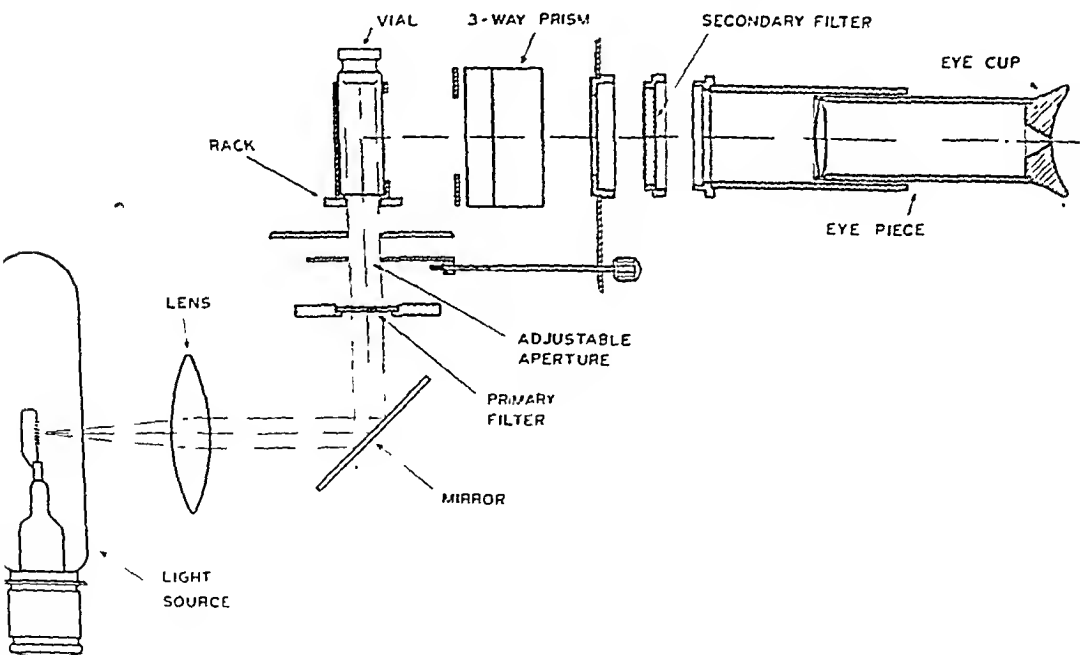


Fig. 1.—Schematic setup of the optical fluorescence comparator.

One of two types of fluorescence meters may be used in this determination: a fluorophotometer of the direct reading type* with special equipment for small samples or a simple visual fluorocomparator recently devised by us.⁸ The fluorocomparator consists of a special incandescent bulb which casts its light from a close distance through a lens and a filter of Corning glass No. 5543 on to a mirror arrangement which reflects the beam on to three matched cuvettes (Fig. 1). The fluorescence thus excited is read through a selective filter of Corning glasses No. 352 plus No. 4303.

*Lumetron No. 402 EF, Photovolt Corporation, New York, N. Y.

The same filter combination is used in the fluorophotometer. This "narrow band" filtering of the secondary filter excludes undesired fluorescence of substances other than atabrine which would otherwise have to be removed by complicated extraction procedures.

The unknown is rapidly read in the instrument against an ether extract containing 0.5 gamma atabrine per cubic centimeter which is set to 100 on the slide wire. This extract is prepared by making a 1:10 dilution of a stock atabrine solution (50 mg. atabrine in 2,000 c.c. of phosphate buffer of pH 7.4). Five cubic centimeters of the diluted stock solution are shaken for four minutes with 25 c.c. absolute ether (C.P.) and separated as described. A blank consisting of an ether extract of the previously mentioned buffer solution alone is set to zero. Since there has been a 1:2 dilution in the preparation of the ether extract, ten divisions on the slide wire now correspond to 0.1 gamma per cubic centimeter.

The accuracy and specificity of the method are shown in Table I, the data for which were obtained by carrying on the following control procedures.

TABLE I

AMOUNT OF ATABRINE ADDED (GAMMA PER CUBIC CENTIMETER)	ATABRINE RECOVERY FROM		ATABRINE CONTENTS OF RESIDUE
	BUFFER SOLUTION (PER CENT)	PLASMA (PER CENT)	
0.5	100	98	0
0.1	97	100	0
0.3	98	97	0
0.2	100	96	0
0.1	98	101	0
0.075	97	97	0
0.05	98	97	0
0.025	102	96	0
0.015	97	102	0
0	0	0	0
0		Plasma icteric	
		0	
0		Plasma lipemic	
		0.015 gamma	
		Unspecific	
		fluorescence	
0		Plasma icteric	
		0	
0		Plasma high	
		protein content	

The watery residue was read after the extraction and was found to give the same result as plain buffer, indicating that no atabrine was left in the residue.

Corresponding amounts of atabrine were added in each instance to 10 c.c. of plasma and 10 c.c. of buffer extracted with the ether. The readings were found to be the same as the readings of the buffer atabrine solution within the range of ± 3 per cent, thus proving the complete recovery from plasma.

Plasma specimens from ten patients with different diseases, especially with high icterus indices and with lipemia, but no atabrine treatment, were extracted and the readings were found to be equal, or very close to equal, to extracts from plain buffer, indicating that the fluorescence obtained with this method is specific for atabrine and atabrine derivatives.

The plasma levels of twenty-two patients suffering from relapses of vivax malaria were studied for their atabrine content during therapy. It was evident after a few observations that the therapeutic plan starting the patients on from 0.3 to 0.6 Gm. of atabrine the first day, followed by 0.2 Gm. daily for seven days, entails an unnecessary delay in the desirable rise in plasma levels (Fig. 2). Although the optimal atabrine plasma level has not been universally accepted, it appears that the maintenance of a plasma level of at least 100 μ g per 1,000 c.c. is

desirable for a therapeutic effect. The initial dose should be large enough to raise the plasma concentration to that height within the first twenty-four hours. Most of the patients obtaining an initial dose of 1.2 Gm., followed by 0.6 Gm. the second day, to be followed by 0.3 Gm. throughout the course, showed this result (Fig. 3). This same experience was described by Shannon and his co-workers.⁹

The skin of patients under atabrine treatment is known to assume frequently a yellowish tint. Since this is related to atabrine deposits in the skin, efforts were made to study the degree of fluorescence by exciting it with ultraviolet radiation of a specific wave length. One may observe to what extent optically active atabrine is deposited in the skin and whether or not this is proportional to the atabrine plasma levels.

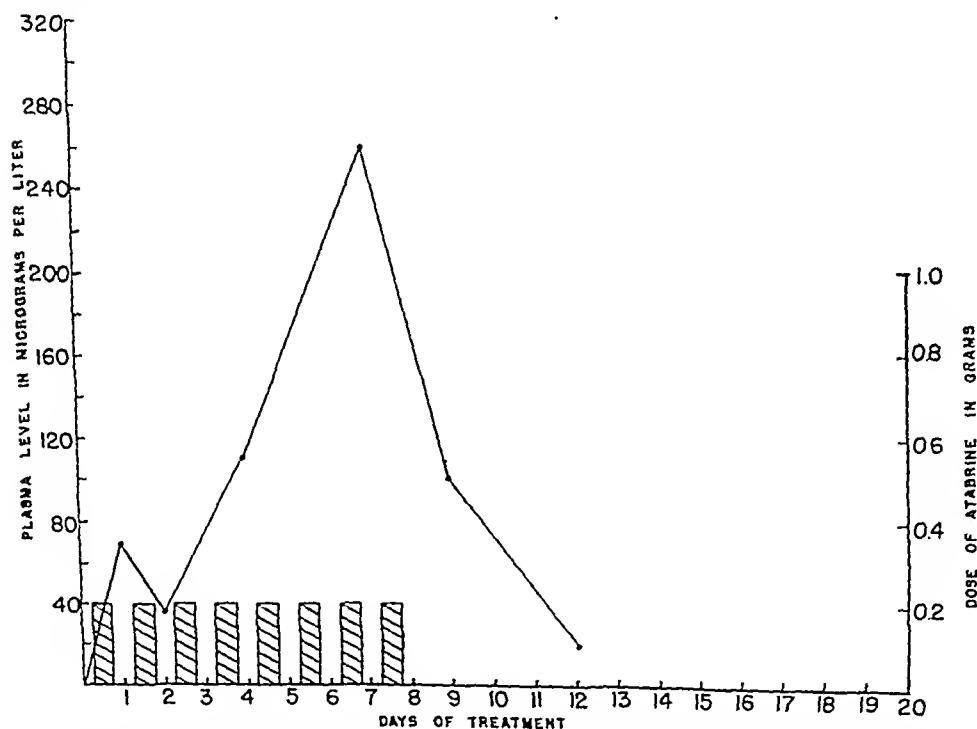


Fig. 2.—L. J. treated according to an old scheme with equal doses of atabrine for seven days. The plasma level rises very slowly, and a satisfactory therapeutic level is obtained only for a short time.

A mercury vapor bulb* is covered with a double filter of Corning glass No. 587. This lamp was used by one of us (K. L.)¹⁰ for fluorescein studies and found to be very effective to demonstrate traces of fluorescence.† If this long-wave ultraviolet light of the main wave length of 3600 Angstrom units is beamed on the skin of the patients during or after atabrine treatment, one will discover that many of them, especially under extended treatment, show a specific greenish-white, jadelike fluorescence of the nails, the palms of the hands, and the plantae

*EH 4 General Electric.

†Supplied by G. W. Gates and Co., Franklin Square, L. I.

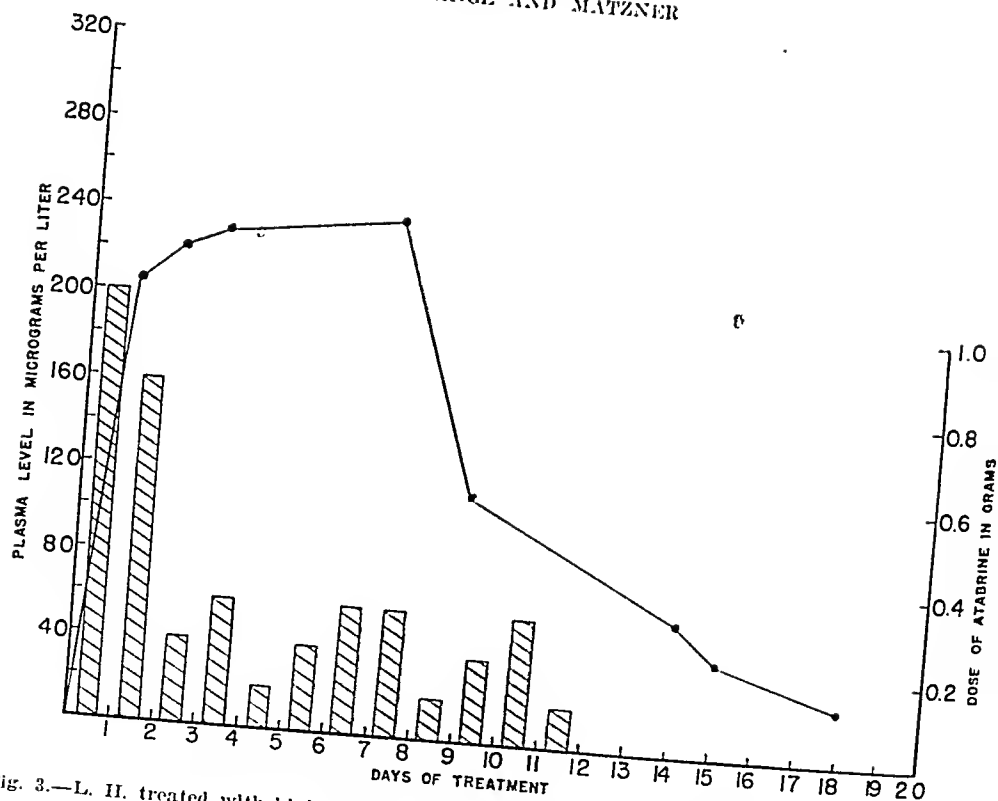


Fig. 3.—L. II. treated with high initial doses. The plasma level rises rapidly to a therapeutic range and stays there for several days.

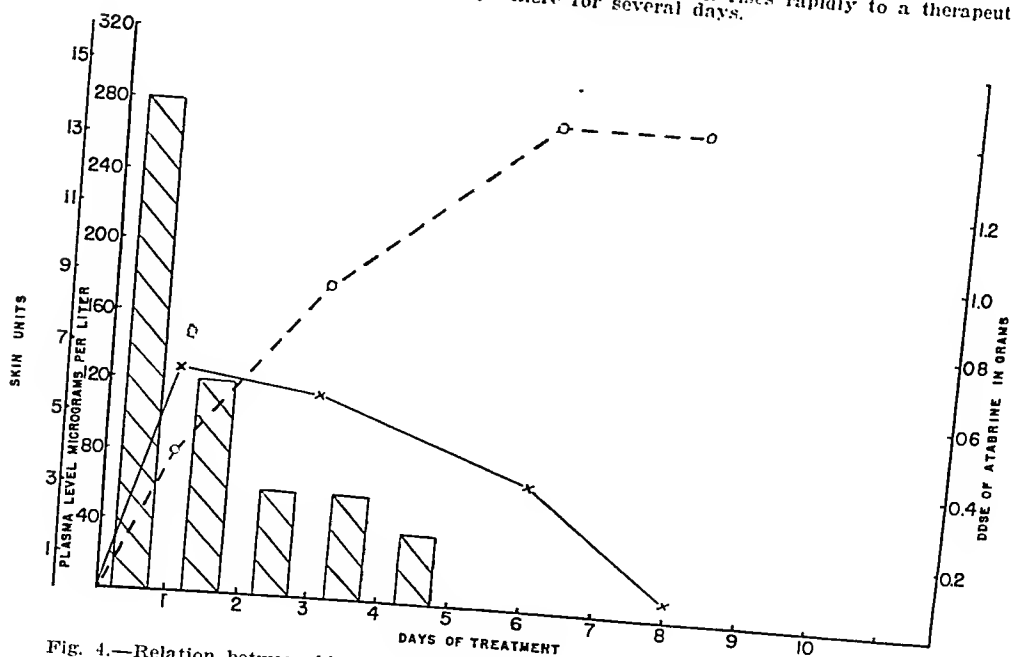


Fig. 4.—Relation between blood and skin levels of atabrine during atabrine therapy.

of the feet, while the skin itself appears in a verdigris color. Once this typical color is familiar to the observer, he can immediately identify the presence and the degree of fluorescence due to atabrine in the skin. The degree of the fluorescence can be measured quantitatively with the Dermofluorometer which we described earlier.⁷ This photoelectric instrument specifically registers the fluorescence in the skin, and the amount of atabrine in the skin can thus be measured quantitatively.

In all twenty-two patients in whom the atabrine plasma level and the skin level were determined daily or at least every other day, no direct correlation between those two factors could be established. While the atabrine content in the plasma rose to its highest levels on the second or third day of therapy, the skin level in most patients rose slowly day by day as long as the therapy was continued. (Fig. 4.) It even continued to rise in the majority of patients for from two to five days after discontinuation of the drug, while the plasma level returned completely or almost completely to zero. It is, therefore, evident that the skin level of atabrine, as determined by the Dermofluorometer, does not correspond to the absolute concentration of atabrine in the blood. We have, however, never seen a case in which the skin level did not rise considerably under therapy. It is not necessary to use the Dermofluorometer to establish the presence of atabrine in the skin, for it can easily be detected visually by the use of the long-wave ultraviolet lamp. The presence of skin fluorescence as an isolated observation does not permit one to differentiate whether it has resulted from present or past administration.

During therapy atabrine is deposited through the matrix into the nail substance. The nail thus assumes an intense jadelike color under ultraviolet light if it contains atabrine. By observing the distribution of fluorescence in the nail, one is able to infer the periods during which atabrine therapy was employed. The amounts given with suppressive treatment are fully sufficient to produce the fluorescence of the nail. With cessation of therapy the blood level drops to zero and deposition of the drug in the nail ceases, thus permitting the nail which is just deposited under the matrix to be free of the specific atabrine color. Thus in an individual who has gone through several courses of therapy or suppressive treatment, one is able to see on the nails, under ultraviolet light, bands of jadelike color in comparison with the purple appearance of the normal nail. Knowing the approximate speed of growth of the nail, one can estimate how long a person has taken the drug and how long he has discontinued taking it.

The specific fluorescence of the nails under long-wave ultraviolet light permits one to state whether or not a person is taking the drug, since in all persons taking atabrine, a deposit will appear in the nails. This would be of importance in regular checks to establish whether the personnel is willfully failing to take the ordered medication.

SUMMARY

1. A rapid simple method to determine atabrine levels in the plasma for the effective treatment and possible suppression of malaria is desirable.

2. A simple extraction method for atabrine from plasma using ether, which can be carried out without elaborate laboratory facilities, is described.

3. A previously described fluorocomparator was found satisfactory for clinical estimations of the plasma level of atabrine.

4. Skin levels of atabrine can be estimated visually by means of a special small ultraviolet lamp or measured quantitatively by a photoelectric Dermo-fluorometer.

5. Clinical studies in twenty-three patients under atabrine therapy reveal that:

(a) There is no direct correlation of skin and plasma levels of atabrine.

(b) The skin level still rises when the blood level has almost returned to zero.

(c) A different scheme of dosage as suggested originally consisting of high initial doses brings about a more satisfactory curve of blood levels.

(d) A deposition of atabrine in the nail, detectable by jadelike fluorescence subsequent to its suppressive or therapeutic administration can be observed.

(e) A study of the nail fluorescence discloses whether and with what regularity atabrine has been taken. The presence or absence of zones of fluorescence in the nails was indicative of the administration or omission of atabrine.

This simple objective observation enables the examiner to establish the consistency with which the drug was taken under suppressive treatment.

We wish to take this opportunity to acknowledge our indebtedness to Rear Admiral Edward U. Reed, Medical Corps, United States Navy, District Medical Officer of the Third Naval District, for his stimulation and cooperation, and to the officers of the St. Albans Naval Hospital, for their kind collaboration.

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PYRIBENZAMINE (N'-PYRIDYL-N'-BENZYL-N-DIMETHYLETHYLENE-DIAMINE HCl), AN ANTAGONIST OF HISTAMINE

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PREVIOUS reports¹⁻⁴ have indicated briefly some of the antihistaminic potentialities of Pyribenzamine; this contribution extends some of the pharmacologic facts gained since these earlier publications and also presents data on toxicologic studies.

Toxicity.—

Acute: Acute toxicity was studied in various species of animals in which the drug was administered by various routes. In Table I data on the toxicity of pyribenzamine are given.

Death was usually preceded by marked excitability and convulsions; in some animals a secondary depression, varying from minutes to an hour or more, intervened between the period of excitation and death. In dogs which recovered from convulsions after 3 mg. or more intravenously, marked excitement and salivation gradually diminished during the next thirty to sixty minutes until complete recovery ensued.

Of special interest is the fact that castration of female rats rendered them less susceptible to the toxic and lethal effects of pyribenzamine; in fact, the LD₅₀ of both sexes was then practically identical. Estrogen therapy of such castrate females was instituted with the anticipation that it might restore them to their previous greater vulnerability; the effect of such therapy is still a moot point.

Preliminary experiments demonstrated that pyribenzamine suspended in a beeswax*-sesame oil mixture according to Code and Varco⁵ was well tolerated subcutaneously by guinea pigs in doses up to 50 mg. per kilogram; with higher doses an increasing number of deaths occurred from twelve to twenty-four hours after injection; with 150 mg. per kilogram the animals died within one and one-half hours after treatment.

Chronic: White rats of both sexes were given daily oral injections of pyribenzamine, 5 mg. per kilogram, and after five months there were no significant changes in erythrocytes, white cells and their differential distribution, hematocrit values, body weight, appetite, reproductive capacities, gastrointestinal functions and general appearance and comport.⁶ These results agree with those of Koepf, Arbesman, and Munafo⁷ concerning the relative safety of this compound after prolonged feeding.

Local: In some rats which survived acute toxicity studies, local necrosis of the skin developed over the site of subcutaneous injection. This was particularly

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*Ten per cent.

TABLE I. LD₅₀ OF PYRIBENZAMINE IN VARIOUS SPECIES ADMINISTERED BY DIFFERENT ROUTES

	NUMBER OF ANIMALS	ORAL (MG./KG.)	NUMBER OF ANIMALS	SUBCU- TANEOUS (MG./KG.)	NUMBER OF ANIMALS	INTRA- VE- NOUS (MG./KG.)
Mice	50	210	300	75	40	12
Rats						
Male	70	570	150	340	50	12
Female	70	515	90	225	35	12
Rabbits			32	33	36	9
Dogs					10	3-4*

*This does not represent LD₅₀ but maximally tolerated dosages of pyribenzamine with recovery.

true in those animals receiving the larger amounts. This was not easily avoided because constant volume was desirable in order to minimize variability of absorption from injected areas of different size. Consequently, concentrations up to 30 per cent of pyribenzamine were required in order to establish the LD₅₀ and such concentrations might well be necrotizing, either because of the general protoplasmic poisoning feature common to all substances known to have local anesthetic properties or because of vasoconstrictor effects which some locally-acting anesthetic agents may exert.

Intramuscular injections of pyribenzamine, 0.1 c.c. of 5 per cent, in the gastrocnemii of rats resulted in replacement of muscle cells by fibroblasts⁸; this result supports the suggestion that local necrosis may follow injudicious use of high concentrations of this drug hypodermically.

Oral in Man: Doses of 100 mg. in tablet form have been generally well tolerated by six human subjects, up to a daily total dosage of 500 mg. Minor complaints in two subjects, but not consistently present, included mild sedation in one and a sense of "pelvic heaviness" with mild nausea in another. It is probable, however, that this higher range of dosage would be more than adequate for the average clinical patient.⁹

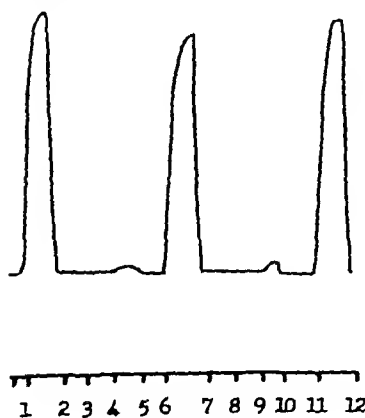


Fig. 1.

Fig. 1.—Intestinal strip from a guinea pig. Histamine, 1 μ g, at 1, 4, 6, 9, 11; pyribenzamine, 0.01 μ g, at 3 and 8; wash at 2, 5, 7, 10, and 12.

Fig. 2.—Studies of the perfused, excised guinea pig lung. The antihistaminic effect of pyribenzamine (N'-pyridyl-N'-benzyl-N-dimethylethylenediamine HCl), 0.025 mg., against histamine phosphate, 0.050 mg.

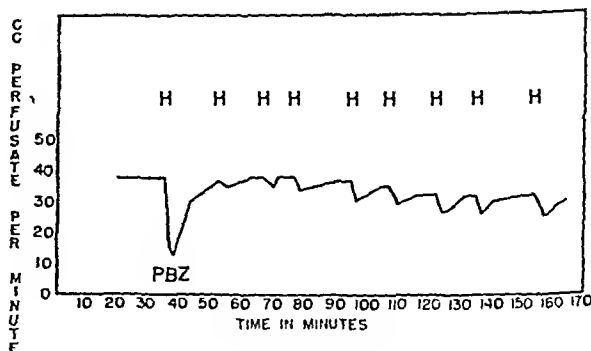


Fig. 2.

*We are indebted to Dr. James Leathem of the Department of Biology of Rutgers University, New Brunswick, N. J., for these pathologic findings.

Antihistaminic Properties (in Vitro).—

Intestine of Guinea Pig: Isolated segments of the jejunum responded to histamine by marked contraction. Much smaller doses of histamine are effective, but 1 μg was uniformly employed in order that some preliminary quantitative appraisal might be made of pyribenzamine in terms of antihistaminic potency. Although 0.02 μg . of pyribenzamine consistently inhibited the contractile response of the intestine to histamine, as little as 0.01 μg was frequently as effective (Fig. 1). Evaluation of the antihistaminic potency of pyribenzamine is now being determined quantitatively, with reference to the protection it affords against histamine hypotension in anesthetized dogs.

Uterus of Guinea Pig: Contraction of this muscle as produced by histamine, from 10 to 20 μg , was nullified by pyribenzamine, from 10 to 50 μg .

Bronchial Muscle of Guinea Pig: Excised lungs, perfused according to the modification of Langendorff's technique described by Tainter, Pedden, and James,⁹ responded well to histamine by contraction of their bronchial muscle. The contractile effect of histamine, 50 μg was usually markedly arrested by pyribenzamine, 25 μg (Fig. 2), and recovery was not complete until after forty to ninety minutes or more.

CONCLUSIONS AND SUMMARY

The toxicity and antihistaminic actions of pyribenzamine are of such an order that extended investigations seem warranted.

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STUDIES ON THE TOXICITY OF SULFONAMIDE DRUGS

I. PORPHYRIN-EXCRETION BY A PATIENT TREATED WITH SULFADIAZINE AND LATER WITH SULFANILAMIDE

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SOON after the introduction of sulfanilamide, it was noted that the administration of this drug gave rise to an increased excretion of urinary porphyrin in patients developing photosensitivity.¹ At about the same time, Rimington and Hemmings^{2, 3} found an increase in the excretion of urinary and fecal coproporphyrin III and small quantities of coproporphyrin I in all patients and animals treated with sulfanilamide. In both of these investigations an attempt was made to relate some of the toxic symptoms of sulfanilamide to the increased production and excretion of porphyrin.

In preliminary attempts to verify these reports, the urine samples in hospital refrigerators were examined in a dark room in near ultraviolet light⁴ to see if any of these were red fluorescent. Red fluorescent urines were not observed, but red or pink fluorescent precipitates were easily and frequently seen. The names and numbers on forty urine bottles containing red fluorescent precipitates were recorded over a period of weeks, and a check made on the hospital charts showed that the corresponding patients had received sulfonamide drugs in all but one instance. The exception was a patient with Hodgkin's disease who had received roentgen-ray therapy. This fluorescence examination of urines was carried out in 1942-1943, at which time sulfapyridine and sulfathiazole were popular. Many patients who were treated with sulfonamides could not be detected by this simple fluorescence test, probably because the urinary porphyrin excretion was not much higher than normal. Most of the urine samples with red fluorescent precipitates were from patients who had received sulfanilamide, but some sulfapyridine- and sulfathiazole-treated patients were detected by means of the red fluorescent urine sediment. This suggested the possibility that these abnormal porphyrins occurring in variable concentrations might be responsible for the bacteriostatic influence of the sulfonamide drugs. This idea had also been entertained and discarded by Rimington and Hemmings.³ The reinvestigation of this problem confirmed his conclusions that the sulfonamide-induced porphyrins probably have no connection with the therapeutic effect but rather appear to be linked with photosensitivity, anuria, neurologic symptoms, and perhaps other toxic effects.

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One of the questions which arose was this: Do the various sulfonamides give rise to the same porphyrin or are specific porphyrins produced by each sulfonamide? A definite answer to this question might indicate whether the excess porphyrins were involved in the specific therapeutic effects or in the toxic effects of sulfonamides. The recent note on the inhibition of bacterial growth by coproporphyrin, hematoporphyrin, and deuteroporphyrin was of interest in this connection.⁵

In this paper it will be shown that different sulfonamides always gave rise to excess amounts of only coproporphyrin III in the same individual.

Because of the laborious procedures involved in extraction and identification of porphyrins in daily collections of urine, it was decided to investigate this problem carefully in one individual who was treated with various sulfonamides at different times. The patient was a 42-year-old white male farmer who came to the hospital April 10, 1943, with empyema on the right side. He was treated for five days with sulfadiazine (6 Gm. per day) (see Fig. 1). On

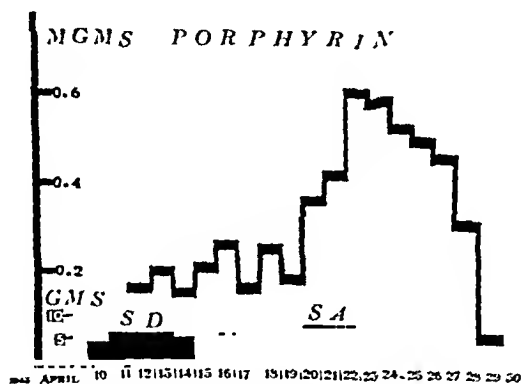


Fig. 1.—Coproporphyrin III excretion after treatment with sulfadiazine and sulfanilamide. Chart, showing the daily excretion of coproporphyrin III with varying dosage of sulfadiazine followed by sulfanilamide. Note the great increase observed when the sulfanilamide was increased to 8 Gm. per day.

the sixth day no sulfonamide drugs were administered. Sulfanilamide was then started. For the next four days the patient received 6 Gm. of sulfanilamide per day, at which time the dosage was increased to 8 Gm. per day and maintained at this level for six more days (see Fig. 1). Thus a total of 27 Gm. of sulfadiazine and 74 Gm. of sulfanilamide were given in fifteen days. During this time the urine was collected over twenty-four hour periods for the determination of the urinary porphyrins. Dobriner's methods of extraction of the urine were employed.⁶ The porphyrins isolated were identified by the following methods:

1. The determination of absorption bands by means of a large spectroscope with a long wave length scale
2. The determination of the HCl number
3. The solubility in 20 per cent NaOH, chloroform, and ether
4. The melting point of the methyl ester

The quantity of porphyrin excreted daily was determined by measuring the absorption of a beam of light (4050 \AA°) isolated by a set of Corning filter glasses,* with a very sensitive colorimeter.† It was found possible to determine accurately the concentration of standard solutions of various porphyrins containing from 0.1 to 1.0 μg per cubic centimeter. Lower concentrations may be determined by using the same instrument as a fluorescence meter, but because this technique involves the careful control of a number of factors (temperature, purity, and HCl concentration), this method has been found to be tedious and less accurate than the colorimetric method which uses light with a wave length of from 4000 to 4100 \AA° .

The quantities of porphyrin excreted daily have been depicted graphically with the dosage of sulfonamides shown in Fig. 1. The collection of urine was not started until the third day of treatment with sulfadiazine. Four daily collections were extracted which contained enough sulfadiazine-induced porphyrin to identify as coproporphyrin III. The urine collected during the eleven days that the patient was treated with sulfanilamide contained far greater amounts of coproporphyrin III. The identification was carried out in the following manner: An attempt was made to fractionate the porphyrin obtained by extraction from ether with increasing concentrations of hydrochloric acid. Most of the porphyrin was extracted with 0.1 per cent hydrochloric acid. The remaining porphyrin appeared in the 0.6 per cent fraction. However, when both fractions were passed back into ether, they had identical absorption spectra which coincided with a sample of synthetic coproporphyrin I and with that recorded for coproporphyrin by other investigators.⁷ When the various fractions of the porphyrin were extracted from the ether with 25 per cent hydrochloric acid, the absorption spectra of all fractions were identical. The position of the middle of the absorption bands on the wave length scale were as follows (stated in millimicrons): in ether, 623.8, 597.2, 577.2, 568.2, 528.4, 495.0; in 25 per cent hydrochloric acid, the bands were located at 593.2, 572.6, 550.3. The porphyrin extracted from the urine of the patient during the time that sulfanilamide was administered had the same absorption bands and solubility characteristics as that extracted from the urine during the period of sulfadiazine treatment. None of the porphyrin extracted was soluble in chloroform, but it dissolved in 20 per cent sodium hydroxide. To see if this was coproporphyrin I or II, the various porphyrin fractions were then pooled and converted into the methyl ester to determine the melting point. The absorption spectrum of the methyl ester in ether was determined on a spectrophotometer. The bands were located at 625.9, 597.5, 570.0, 528.0, 494.0. The melting point of the methyl ester crystals was from 145.2 to 147° C. When remelted the crystals melted at from 165 to 168° C. Therefore, we concluded that the porphyrin was coproporphyrin III.

The fact that the same porphyrin is excreted when one individual is treated with different sulfonamides should have been expected on the basis of the investigations of Rimington and Hemmings.³ They tested a number of

*Corning 3060, 430, 385.

†Lumetron 402 EF.

compounds resembling sulfanilamide or prontosil and found the porphyrin-inducing property related to the phenylamine group. Aniline and similar substances were found to induce porphyrinuria. Since the phenylamine group is essential for therapeutic activity, it is not modified in most of the sulfonamides. They should, according to Rimington's hypothesis, all undergo oxidation in vivo and in turn oxidize hemoglobin to methemoglobin which is degraded perhaps by way of hematin to coproporphyrin III instead of bilirubin. If this process is responsible for the toxic effects of sulfonamides, then all the sulfonamides commonly used should present toxic symptoms of the same type and degree. Such is, however, not the case.

A survey of the literature revealed that, in addition to Brunsting¹ and Rimington and Hemmings,² other investigators had reported porphyrinuria resulting from sulfonamide treatment. Long and Bliss,³ in a discussion of the toxic manifestations of sulfanilamide, stated that approximately 3 per cent of the patients exhibited porphyrinuria. Wien⁴ confirmed Rimington and Hemming's observation that sulfanilamide administration causes porphyrinuria in the human subject and in rats, but he claimed that sulfapyridine did not significantly alter the rate of porphyrin excretion in rats. The values which Wien gave as normal for rats ranged from 24 to 48 μg per rat per day. The range found for sulfapyridine-treated rats was from 36 to 84 μg . The rats given sulfanilamide excreted from 90 to 260 μg daily. During this same year other investigators¹⁰ found normal urinary porphyrin values in several patients who had taken sulfanilamide, and one of these was light sensitive. The methods used to extract and determine the porphyrin in this case are open to question. Small quantities of urine were extracted, and the relatively crude and impure porphyrin extracts were roughly estimated by visual fluorometry. More recently, Sehreus and Kreig¹¹ determined the porphyrin excretion in thirteen patients with either gonorrhea or pyoderma treated with various sulfonamides. Eight of the thirteen showed slight increases in porphyrin excretion. This gives the impression that a marked increase such as we have observed (from 200 to 600 μg per day) occurs only sporadically in sulfonamide-treated patients. This is doubtlessly true, and the amount of porphyrin excreted in any given case is probably determined by the type of sulfonamide given, the dosage, the individual susceptibility or condition of the patient, and perhaps the disease.

The possibility that some of the severe toxic reactions which occur infrequently are limited to those patients who respond to sulfonamides by producing and excreting relatively large amounts of porphyrin deserves further investigation.

SUMMARY AND CONCLUSIONS

The fact that sulfonamide therapy frequently gives rise to a mild and variable porphyrinuria has been confirmed by semiquantitative observations on forty patients. In a careful study of the urinary porphyrins of one of these, it was observed that the administration of either sulfadiazine or sulfanilamide to the same patient at different times gave rise to the excretion of

abnormal amounts of the same kind and type of porphyrin, coproporphyrin III. The amount of porphyrin excreted during sulfadiazine treatment was less than when a similar dose of sulfanilamide was administered. The amounts of porphyrin excreted in this case paralleled the relative toxicity of the two drugs. Since aniline, lead salts, sulfonal, and other similar compounds give rise to porphyrinuria, but presumably have no bacteriostatic action, the coproporphyrin III is probably one of the coincidental by-products of sulfonamide action.

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OBSERVATIONS ON THE ACTIVITY IN VITRO OF SUCCINYL-SULFATHIAZOLE AND PHTHALYLSULFATHIAZOLE

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THE recent studies by Poth and co-workers^{1,2} on a series of N⁴-dicarboxylic acid-substituted sulfonamides synthesized by Moore and Miller³ resulted in the development of two new sulfonamides, succinylsulfathiazole and phthalylsulfathiazole, which have found extensive use as intestinal antiseptics. Theoretically, these two N⁴-acyl derivatives of sulfathiazole should have no activity in vitro. However, the few reports in the literature regarding this property have been conflicting. Kirby and Rantz⁴ found that succinylsulfathiazole had practically no activity in vitro. Poth and associates¹ reported slight bacteriostatic activity for succinylsulfathiazole against *Escherichia coli* in vitro but thought that the activity shown might have been due to a small amount of free sulfathiazole present in the sample studied. In a later report, however, Poth and Ross⁵ used data obtained in vitro with succinylsulfathiazole in a comparison of results obtained with a number of carboxylic acid derivatives of sulfathiazole. Brewer⁶ was also able to demonstrate activity in vitro for succinylsulfathiazole. With *Shigella sonnei* as the test organism, the activity was found to be directly proportional to the amount of drug present and inversely proportional to the number of organisms. In comparison, sulfaguanidine had no activity against the same organism. Schweinberg and Yetwin⁷ observed that phthalylsulfathiazole had a better bactericidal action than sulfathiazole against *E. typhosa* and *Shigella paradyesenteriae* (var. Flexner), while sulfathiazole was the more effective of the two drugs against *E. coli*, *Shigella dysenteriae* (Shiga) and the paradyesenteriae strain of Hiss.

Evidence is presented here in an attempt to show that demonstrable activity in vitro for both succinylsulfathiazole and phthalylsulfathiazole is due not to each drug per se but apparently to free sulfathiazole which not only may be present as a contaminant in the original material but can be augmented in concentration as a result of decomposition of the conjugated sulfathiazole during preparation for testing.

EXPERIMENTAL

As a preliminary to the studies in vitro, a number of solutions of succinylsulfathiazole and phthalylsulfathiazole† were prepared at different concentrations in distilled water buffered at pH 7.0.‡ The drugs were dissolved at room temperature and then analyzed for free diazotizable substance after the method

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†Samples of the drugs were purchased from the open market.

‡The buffered water (pH 7.0) was prepared by the addition of 6.805 Gm. of KH₂PO₄ and 29.63 c.c. of 1 N NaOH to distilled water to make 1 liter of solution.

of Bratton and Marshall.⁶ Aliquots of the solutions were autoclaved at 15 pounds pressure for varying periods of time, and diazotization was again performed on the respective solutions. Because of the extreme rapidity with which phthalylsulfathiazole hydrolyzes in acid solution, it was necessary to modify the diazotization procedure somewhat in order to obtain reproducible results. Typical findings from these analyses are shown in Table I. The results indicated that the samples of succinylsulfathiazole and phthalylsulfathiazole studied were contaminated with a substance capable of diazotization. It was also observed that the amount of this substance could be increased by heating, presumably as a result of decomposition of the original N'-acylated sulfathiazole derivatives.

TABLE I. FREE DIAZOTIZABLE SUBSTANCE IN SOLUTIONS OF SUCCINYL-SULFATHIAZOLE AND PHTHALYL-SULFATHIAZOLE BEFORE AND AFTER AUTOCLAVING

CONCENTRATION OF DRUG BY WEIGHT (MG. %)	TREATMENT	DIAZOTIZABLE SUBSTANCE* IN SUCCINYL-SULFATHIAZOLE		DIAZOTIZABLE SUBSTANCE* IN PHTHALYL-SULFATHIAZOLE	
		CONCENTRATION (MG. %)	PER CENT OF TOTAL WEIGHT	CONCENTRATION (MG. %)	PER CENT OF TOTAL WEIGHT
16	Unheated			0.19	1.19
	5 min. heat			1.26	7.88
	20 min. heat			2.50	15.63
32	Unheated	0.10	0.31	0.33	1.03
	5 min. heat	0.18	0.56	2.74	8.56
	20 min. heat	0.30	0.94	5.55	17.34
64	Unheated	0.19	0.30	0.62	0.97
	5 min. heat	0.40	0.63	5.15	8.10
	20 min. heat	0.61	1.00	9.80	15.31
128	Unheated	0.31	0.24		
	5 min. heat	0.62	0.48		
	20 min. heat	1.02	0.80		

*Calculated as free sulfathiazole.

TABLE II. HYDROGEN ION CONCENTRATIONS OF SOLUTIONS OF SUCCINYL-SULFATHIAZOLE AND PHTHALYL-SULFATHIAZOLE PREPARED IN WATER BUFFERED AT pH 7.0

CONCENTRATION OF SOLUTION (MG. %)	DRUG*	HYDROGEN-ION CONCENTRATION (pH)		
		INITIAL	AFTER 5 MIN. HEAT	AFTER 20 MIN. HEAT
16	PST	7.00	7.00	7.00
	SST	6.98	6.98	6.98
32	PST	6.97	6.97	6.97
	SST	6.93	6.93	6.93
64	PST	6.93	6.93	6.93
	SST	6.86	6.86	6.86

*PST, Phthalylsulfathiazole; SST, succinylsulfathiazole.

As a check on the buffering capacity of the diluent used for the various solutions, the pH of each solution was determined immediately upon dissolution of drug and after autoclaving. No marked change from the original pH of the buffered water was indicated when solutions of drug as high as 128 mg. per 100 c.c. were prepared. The initial hydrogen ion concentrations of all solutions remained unaltered when heated at 15 pounds pressure for twenty minutes (Table II).

On the assumption that the varying degrees of diazotization were due to the presence of free sulfathiazole, the following procedure was used to test the hypothesis.

Solutions of succinylsulfathiazole and phthalylsulfathiazole were prepared in a synthetic medium consisting of asparagine, glucose, and inorganic salts.⁹ Concentrations for the various tests ranged from 32 to 512 mg. per cent by weight. All solutions were made up at room temperature, and the amount of free diazotizable substance was determined for each. An aliquot of each solution was then sterilized by Seitz filtration while a second and third portion were autoclaved at 15 pounds pressure for five and twenty minutes, respectively. The Bratton and Marshall reaction was again carried out on the Seitz filtrates and heated portions. Solutions of sulfathiazole were next prepared in the synthetic medium to contain concentrations of the drug comparable to the respective amounts of free diazotizable substance (calculated as sulfathiazole) in each of the variously treated solutions of succinylsulfathiazole and phthalylsulfathiazole. All sulfathiazole solutions were checked by the color reaction before use, and each of these corresponding to the respective solutions of the N⁴-acylated derivatives was similarly treated; that is, one portion was passed through a Seitz filter and a second was autoclaved for five minutes, a third for twenty minutes. This procedure was followed throughout, even though filtration and heating had no apparent effect whatever on the initial concentrations of sulfathiazole.

The comparison in vitro of the conjugated derivatives of sulfathiazole with free sulfathiazole was performed by serially diluting, aseptically, each initial drug solution by halves in plain sterile synthetic medium and inoculating with *E. coli*. Each tube contained a final volume of 5.0 c.c. of medium plus drug at the various dilutions, and 0.1 c.c. of a diluted eighteen-hour culture was added so that the initial number of organisms averaged about 500 per cubic centimeter. The tubes were incubated at 37° C. and examined for visible growth at twenty-four hour intervals.

RESULTS

It was apparent from the data obtained that succinylsulfathiazole and phthalylsulfathiazole exerted inhibitory action against *E. coli* under the conditions of the test. However, when free sulfathiazole was tested at concentrations corresponding to those of the free diazotizable substance present in the N⁴-acylated sulfathiazole solutions, identical end points resulted. For example, a concentration of 32 mg. per 100 c.c. of succinylsulfathiazole (Seitz-filtered) showed complete stasis, while partial inhibition of *E. coli* was affected at 16 mg. per 100 c.c. The amount of free diazotizable substance at each of these concentrations of succinylsulfathiazole was equivalent to 0.08 and 0.04 mg. of sulfathiazole per 100 c.c., respectively, at which concentrations free sulfathiazole produced the same results (Table III). When an aliquot of the same initial solution of succinylsulfathiazole was autoclaved for twenty minutes, complete stasis occurred at a concentration of 4.0 mg. per 100 c.c., at which level 0.06 mg. per 100 c.c. was present as free diazotizable substance. A corresponding amount of free sulfathiazole (0.06 mg. per 100 c.c.) likewise showed complete

inhibition of growth of the test organism. The protocol of an experiment illustrating a similar comparison of activity with phthalylsulfathiazole and sulfathiazole is given in Table IV.

DISCUSSION

It is believed that the apparent activity in vitro shown by succinylsulfathiazole and phthalylsulfathiazole has been identified with that of free sulfathiazole. Hydrolysis of either of these N^4 -acylated compounds would yield sulfathiazole and the respective dibasic acid. The possibility of any antibacterial activity due to either succinic acid or phthalic acid alone was ruled out, however, since tests in vitro showed them to have no effect on *E. coli* in concentrations much higher even than those expected through decomposition.

The exact bearing that these findings may have in relation to the in vivo mode of action of succinylsulfathiazole and phthalylsulfathiazole cannot be stated conclusively at this time. Poth and associates¹ originally believed that these drugs might owe their antibacterial activity to free sulfathiazole liberated by hydrolysis of the acylated derivatives. It was concluded from later work,^{10, 11} however, that this alone could not fully explain their action and that a large portion of the activity of succinylsulfathiazole and phthalylsulfathiazole must consequently be attributed to the conjugated molecule.

The bacteriostatic effectiveness of sulfanilamide derivatives has been associated with the free amino group situated in the para position of the benzene nucleus. Activity may be destroyed through conjugation in the body or by chemical substitution otherwise. This fact becomes of importance in the study of the behavior in vitro of certain derivatives of the parent sulfonamides, especially N^4 -acylated compounds. Caution should be exercised, therefore, in ascertaining first of all the purity of such compounds and secondly whether the substances are stable enough to withstand the various steps involved in their preparation for assay.

SUMMARY

1. Data are presented showing that succinylsulfathiazole and phthalylsulfathiazole may contain a diazotizable substance as an impurity. The amount of this substance can be progressively increased by prolonged heating of the N^4 -acylated derivatives of sulfathiazole in solution.
2. It is concluded that the impurity is sulfathiazole and that its presence accounts for any in vitro activity shown by the conjugated sulfathiazoles.

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FATE OF SODIUM RICINOLEATE AFTER ORAL ADMINISTRATION TO WHITE RATS

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MUCH has been published with regard to the properties of sodium ricinoleate, but little is known of its action in the intestinal tract when it is used in the treatment of certain chronic intestinal disorders. It is believed that the therapeutic action may be due to detoxication of many intestinal toxins or to alteration of the intestinal flora.¹⁻⁴ Solutions of sodium ricinoleate are capable of neutralizing various bacterial toxins, phytotoxins, and cobra venom in vitro and possibly in the living animal.^{2, 5-9} Some of the detoxicated toxins when injected into various animals have produced immunity against many times the lethal dose of the respective toxins. Consideration has also been given to its bactericidal or bacteriostatic action on many organisms. Kolmer⁹ found sodium ricinoleate to have a low bactericidal activity in the test tube.

As yet there are few facts upon which to base an adequate explanation of the therapeutic action of sodium ricinoleate in the intestinal tract.

In an earlier study¹⁰ no evidence was obtained that ricinoleate soaps or castor oil are absorbed from the intestinal tract, as determined by chylomicron count or by absorption of sudan IV. Paul and McCay¹¹ fed guinea pigs a diet containing 6 per cent ricinoleic acid and found from 91 to 93 per cent utilized as determined by fecal analyses. Castor oil, when present in the diet at a level of 6 per cent, was utilized to the extent of 92 per cent in rabbits and 99 per cent in sheep. They believe that melting point rather than degree of saturation is the determining factor in the utilization of fats in guinea pigs.

Recently Stewart and Sinclair¹² found the amount of fats excreted in rats on a diet containing 48.4 per cent castor oil to be about 2 per cent of the intake. They concluded that ricinoleic acid is readily metabolized, as there was no increase in the acetyl number of fecal fat over the control values and no evidence of its presence in the phospholipids of the small intestine, liver and muscle, or glycerides of the liver. Of the fatty acids of depot fat, about 7 per cent was ricinoleic acid.

In the present study sodium ricinoleate was either fed in a diet or administered by stomach tube to white rats, and the amounts absorbed from the gastrointestinal tract or excreted in feces were determined.

EXPERIMENTAL STUDY

The work involved either feeding or administering by stomach tube sodium ricinoleate‡ to white rats. The extraction of fats and fatty acids was made from gastrointestinal contents and feces, with subsequent separation into petroleum

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‡Soricin, The Wm. S. Merrell Brand of Sodium Ricinoleate.

ether (from 40 to 60° C.) and ethyl ether fractions. The fatty acid of sodium ricinoleate has a low solubility in petroleum ether but is completely soluble in ethyl ether. Its high acetyl number is useful for identification. The presence of ricinoleic acid was based on the total amount of fats and fatty acids extracted, amounts extractable by petroleum ether followed by re-extractions with ethyl ether and by acetyl numbers of the two fractions.

Procedure for Carrying Out Excretion Studies.—Sodium ricinoleate was administered to white rats either by stomach tube or by incorporating it into a stock diet.* Those animals receiving it by stomach tube were given 0.4 Gm., and the feces were collected for forty-eight hours. Those animals receiving it in their diet consumed approximately 0.5 Gm. daily for a period of two months. The feces were collected during the entire period and an aliquot portion used for the determinations.

Feces were collected also from control animals not receiving sodium ricinoleate. Recovery tests of sodium ricinoleate added to feces also were made.

Methods for Preparation, Extraction, and Testing of Materials.—All samples of feces were dried at 100° C. for twelve hours, weighed, and then powdered. A suitable aliquot was transferred to a separatory funnel containing 25 c.c. of distilled water. The mixture was made acid with 10 per cent hydrochloric acid, and the total fats and fatty acids were extracted by shaking with 5 c.c. portions of chloroform. The extracts were filtered successively through filter paper into a tared flask. The combined extract was evaporated to dryness on a steam bath under a current of air. The weighed residue was dissolved in 20 c.c. of absolute ethanol and transferred to a separatory funnel. About 10 c.c. of water was added, after which it was washed three times with 30 c.c. portions of petroleum ether. The petroleum ether fraction was washed twice with 15 c.c. portions of 50 per cent ethanol, which was transferred back to the alcoholic solution. The petroleum ether fraction was evaporated to dryness in a tared flask and weighed. Fifty cubic centimeters of ethyl ether and enough distilled water were added to the alcoholic solution to cause separation. The ethyl ether fraction was drawn off, and the aqueous alcoholic mixture was extracted three more times with ethyl ether. The ethyl ether extractions were combined in a tared flask, evaporated to dryness, and weighed.

The acetyl value of each fraction was determined by the method of Roberts and Schuette.¹²

Absorption Studies of Sodium Ricinoleate From Gastrointestinal Tract.—Acute studies were made on the amount of fats and fatty acids found in various segments of the gastrointestinal tract of rats twenty-four hours after oral administration of a large dose of sodium ricinoleate. Eight white rats, all weighing approximately 220 grams each, were used in the experiment. They were without food for from sixteen to twenty-four hours. Four were administered 2 c.c. of distilled water by means of a stomach tube and were used as controls. The other four animals received 400 mg. of sodium ricinoleate in 2 c.c. of distilled water. The feces were collected from each animal. All animals were sac-

*"Harlan Special Rat Ration" obtained from Harlan Small Animal Industry, Cumberland, Ind. The fat content of this diet is 4.4 per cent.

rified twenty-four hours after gastric intubation, and the stomach and intestines were removed. The contents of the stomach and small intestine were washed into a separatory funnel by means of a stream of hot water. The contents of the large intestine and the feces were transferred to another separatory funnel. The aqueous mixtures were acidified with hydrochloric acid and extracted five times with 25 c.c. portions of chloroform. These were filtered successively through chloroform-wetted cotton into a tared evaporating flask. The filtrates were evaporated to dryness on a steam bath and placed in a vacuum desiccator overnight. The flasks were weighed again to determine the total fat by difference.

Similar acute studies were made on another group of rats to determine the amount of lipids remaining in the stomach and various segments of the intestinal tract, one-half, three, six, sixteen, and twenty-four hours after administration of 400 mg. of sodium ricinoleate in 2 c.c. of water by stomach tube. The animals, which weighed approximately 200 grams each, were without food for from sixteen to twenty-four hours before the test. Animal charcoal was given with the sodium ricinoleate to show the distance it had traveled down the intestinal tract at the time each animal was killed.

Three control animals received the animal charcoal in 2 c.c. of water but no sodium ricinoleate. One of these rats was killed one-half hour, another three hours, and the third six hours later. The contents of the stomach, of the upper, and of the lower half of the small intestine of all animals were removed and assayed for the amounts of lipids by the method just described.

RESULTS

The results of the excretion studies are shown in Table I. Under the condition of the experiment approximately 25 per cent of ricinoleic acid is extracted by petroleum ether and 75 per cent by ethyl ether. Under similar experimental conditions the total fat from the control feces was about equally divided between the petroleum ether and ethyl ether fractions. When 300 mg. sodium ricinoleate were added to 2 Gm. of control feces, the total fats and fatty acids recovered were re-extracted with petroleum ether and then with ethyl ether. The amounts of material dissolved in the two solvents were 33 and 65 per cent, respectively, indicating by its solubility that ricinoleic acid was present. The acetyl value for each fraction was considerably higher than that of the respective control sample.

TABLE I. PARTITION OF TOTAL LIPIDS BETWEEN PETROLEUM ETHER AND ETHYL ETHER, TOGETHER WITH ACETYL NUMBERS FOR EACH FRACTION

MATERIAL	NUMBER OF TESTS	TOTAL LIPIDS FOUND IN MATERIAL (PER CENT)	PETROLEUM ETHER FRACTION		ETHYL ETHER FRACTION	
			LIPIDS (PER CENT OF TOTAL)	ACETYL NUMBER	LIPIDS (PER CENT OF TOTAL)	ACETYL NUMBER
Sodium ricinoleate (300 mg.)	2	84.4	24.1	242	74.8	242
Control feces (2 Gm.)	2	6.6	53.4	141	47.6	156
Sodium ricinoleate (300 mg.) added to control feces (2 Gm.)	2	16.3	32.9	218	64.5	219
Feces from animals receiving 0.5 Gm. sodium ricinoleate daily	4	6.0	59.9	128	36.8	142

Values obtained after the administration of a single dose of sodium ricinoleate or after the daily feeding of 0.5 Gm. of the material for two months are quite similar to those obtained with control fees and indicate the absence of ricinoleic acid. This would suggest that sodium ricinoleate either is absorbed from the intestinal tract or is so altered chemically as not to be extracted by the technique used.

In an experiment in which 400 mg. of sodium ricinoleate were administered orally and the rats killed twenty-four hours later, there was no indication that an appreciable amount of the administered substance was present in the gastrointestinal tract. In the control group the average weight of total fat and fatty acids from the contents of the stomach and the small intestine was 42 mg., while that from the large intestine, in combination with the twenty-four hour fecal collection, was 61 mg. After the administration of 400 mg. of sodium ricinoleate, the average values were 49 and 69 mg., respectively. These slight increases account for only about 4 per cent of the dose administered.

TABLE II. RATE OF ABSORPTION OF SODIUM RICINOLEATE FROM GASTROINTESTINAL TRACT (400 MG. OF SODIUM RICINOLEATE ADMINISTERED BY STOMACH TUBE TO FASTING RATS)

TIME AFTER ADMINISTRATION (HR.)	STOMACH (MG.)	AMOUNT OF LIPIDS EXTRACTED			
		SMALL INTESTINE		TOTAL	
		UPPER (MG.)	LOWER (MG.)	(MG.)	(PER CENT OF DOSE)
Control	3	4	4	11	
1/2	260	26	13	299	77
3	239	38	26	303	78
6	185	42	30	257	66
16	26	8	11	45	9
24	8	7	9	24	4

In another experiment determinations were made for amounts of sodium ricinoleate and charcoal remaining in the stomach, in the upper, and in the lower half of the small intestine after varying periods of time following the dose. The amounts extracted are shown in Table II. The amount of lipids found in the control animal was somewhat less than that in the preceding experiment. There was considerable delay in the passage of sodium ricinoleate and charcoal from the stomach into the intestines. The stomach appeared to be considerably bloated during the first six hours after sodium ricinoleate. The charcoal was present only in the stomach and the upper half of the small intestine of animals killed one-half and three hours after the dose was administered. Six hours after the dose it was present also in the lower half of the small intestine. After sixteen hours most of the charcoal had passed into the large intestine, and the stomach was flat and not bloated. No charcoal was present in any segment after twenty-four hours, and the stomach was completely empty.

In the control animals, one-half hour after a dose was administered, there was charcoal present in all three segments. After three hours none of the charcoal was found in the stomach, and most of it was present in the lower half of the small intestine, with only a trace in the upper half. Nearly all had passed into the large intestine after six hours.

DISCUSSION

The data obtained in this study are in accord with previous studies^{11, 12} on ricinoleic acid and castor oil and indicate that sodium ricinoleate is absorbed from the gastrointestinal tract and is not excreted in any appreciable amount in feces. Little information has been found regarding its fate after passing the intestinal barrier. Small amounts may be stored as a glyceride in fat depots, as was shown by Stewart and Sinelair¹² after the administration of castor oil. The major portion undoubtedly is oxidized in a manner similar to that of closely related unsaturated fatty acids. It is not likely that any free sodium ricinoleate occurs in the systemic blood stream at any time. Kolmer⁹ has shown that 1 c.c. of 1:800 sodium ricinoleate produces complete hemolysis of 1 c.c. of defibrinated rabbit blood within twenty minutes at 37° C. Sodium ricinoleate frequently is used as a sclerosing agent for the treatment of varicose veins, due to its powerful hemolytic activity; it forms a jellylike coagulated mass when injected intravenously.¹⁴ Accordingly, a substance with such properties is not likely to occur in a significant concentration in circulating blood after oral administration. Histologic examinations of cross sections of various portions of the intestinal tract, in rats on diets containing 0.5 Gm. sodium ricinoleate per day for periods as long as two months, do not show any tissue damage of any kind. The lack of sclerosing action would seem to indicate that sodium ricinoleate is undoubtedly converted by the epithelial cells into a triglyceride, in a manner similar to that of any other closely related fatty acid, and is thus carried into the lacteals in the form of chyle.

These studies do not explain the beneficial clinical action of sodium ricinoleate administered orally during the treatment of certain intestinal disorders. One cannot overlook the likelihood that it acts locally in the intestinal tract as a stimulant, thus altering intestinal rhythm either directly or through the sympathetic nervous system. Alteration in intestinal flora is also a good possibility, since it is well known that the character of the flora is an important factor for the general health of an individual. Another possibility is its reaction with toxins or poisons which in themselves may act as stimulants for abnormal intestinal rhythm or which may be absorbed into the blood stream, giving rise to the symptoms accompanying such disorders. The concentration of urinary indican in some patients with certain intestinal disturbances is quite high¹⁵; this generally returns to normal immediately after treatment with sodium ricinoleate.

From preliminary studies on the rate of absorption of sodium ricinoleate from different segments of the gastrointestinal tract, it appears that there would be considerable time for it to carry on its function as a therapeutic agent, as the emptying time of the stomach and the rate of absorption from the intestinal tract are considerably prolonged. The rate of passage of an indicator (animal charcoal) along the intestinal tract is considerably delayed in the presence of sodium ricinoleate, but the mechanism of this action has not yet been determined.

SUMMARY

Sodium ricinoleate was administered to white rats either by stomach tube or by feeding approximately 0.5 Gm. per day in the diet for two months. There is no evidence that any ricinoleic acid is excreted in the feces of these animals.

Absorption studies indicate that orally administered sodium ricinoleate is almost completely absorbed from the gastrointestinal tract of rats during a period of twenty-four hours.

The rate of passage of an indicator (animal charcoal) through the intestinal tract of rats is considerably retarded by the oral administration of sodium ricinoleate.

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OBSERVATIONS ON THE EFFECT OF LOWERED OXYGEN TENSION ON SICKLEMLIA AND SICKLE CELL ANEMIA AMONG MILITARY FLYING PERSONNEL

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SURVEYS of large groups of Negroes in the United States reveal that the incidence of erythrocyte sickling varies from 4.3 to 14 per cent, with an accepted average between 7 and 9 per cent.^{1,2} Approximately one of fifteen develops the anemic state. Sick cell anemia, therefore, is considered relatively uncommon when compared with the sickling trait or sicklemlia. Reports on the incidence of the sickling phenomenon in Negroes outside the United States are few; to date, we have found only three such reports³⁻⁵ in medical literature.

Because it has been demonstrated that diminishing the oxygen tension of wet preparations of the blood of patients with sickle cell anemia or sicklemlia will cause the majority of erythrocytes to assume the sickle shape, it appeared important to determine whether the high altitudes reached by Negro flying personnel might increase the degree of sickling in vivo and lead to the development of symptoms. No reports on the effects of diminished oxygen tension in vivo on the sickling phenomenon could be found in the literature.

At the Tuskegee Army Airfield the flying personnel is predominantly Negro. The aviation cadets are all Negroes, and since 1941 pilots comprising fighter and bomber groups have received their preflight, primary, basic, and advanced training at that airfield and near-by Moton Field. On acceptance for aviation training all preaviation cadets undergo thorough physical and psychiatric examinations similar to those given at other fields. These men are a select group, and the percentage of eliminees in both the preflight and flight groups compares favorably with others in the Southeastern Training Command. Searching physical examinations are repeated at regular intervals during the training period; they include chest roentgenograms, serologic tests for syphilis, and urinalyses.

When the Negro was first assigned to aviation training, some doubt was expressed by physicians as to the physical capacity of those men with the sickling trait to tolerate high altitudes. No emphasis was placed on this problem, however, because it was expected that the routine use of oxygen would counteract any undesirable effects resulting from diminished oxygen tension. From the beginning, routine wet preparations of the blood were not obtained, and no incidence of the sickling phenomenon was determined. However, in over 1,500 cadets under training, none have been eliminated because of proved flying deficiencies arising from the sickling trait or active sickle cell anemia.

Early in 1945 we decided to study the incidence of sickling in Negro aviation cadets to determine whether there was any significant difference in the per-

centage of elimination between the groups having the sickling trait and the "normals." A small group of combat returnees was also observed for the sickling trait. It was our opinion that, if the relative incidence of sickling was preserved in various stages of training and in the returnees, this would aid in eliminating the sickling problem as a barrier to high altitude flying. In addition, to investigate further the effects of high altitude on persons with sickle cell anemia, a small group of cadets was selected, on a volunteer basis, for observation while on a simulated flight in the low pressure chamber without oxygen.

Statistical Evidence That the Presence of Sickle Cell Anemia in Flying Personnel Does Not Lead to Their Elimination From the Air Services.—Two hundred sixty cadets in various stages of training were examined for sickling by the wet preparation method. Two preparations were made from capillary blood on each subject. If after forty-eight hours these preparations failed to reveal sickling, they were labeled as negative. Slides were read at the end of twenty-four and forty-eight hours. It has been our experience, as well as that of Sherman,⁶ that too many factors enter into the sickling phenomenon to attempt to gauge correctly the degree or time of appearance of sickling on the wet preparation; therefore, no attempt was made to determine accurately the percentage of sickling on any wet preparations. They were either declared negative or positive. Later, erythrocyte counts, thin blood films, and sedimentation rates were studied in a representative number of these men for evidence of active sickle cell anemia.

TABLE I. INCIDENCE OF SICKLE CELL ANEMIA IN CADETS AND COMBAT RETURNEES; ALL CADETS OBSERVED FOR MINIMAL PERIOD COVERING ONE PHASE OF TRAINING

NUMBER EXAMINED	STAGE OF TRAINING	NUMBER POSITIVE FOR SICKLING	PER CENT SICKLING	NUMBER NEGATIVE ELIM-INEES	PER CENT NEGATIVE ELIM-INEES	NUMBER SICKLING ELIM-INEES	PER CENT SICKLING ELIM-INEES
152	Primary	11	7.24	43	30.5	2	18.2
80	Basic	6	7.5	8	10.6	0	0.00
28	Advanced	2	7.14	0	00.0	0	0.00
52	Returnees	4	7.7	—	—	—	—
312		23	7.37	51	23.7	2	11.76

All cadets in our series were observed for a minimal period of three months. Of the 260 cadets examined, 152 were beginning their primary training. Another twenty-eight men were examined at the time of the last physical examination prior to receiving their commissions; the other 80 were in basic training. In addition to the 260 cadets examined, an additional fifty-two combat returnees were examined by the wet preparation method.

The results of our survey are presented in Table I. Note that a comparable incidence of sickling is preserved throughout all groups studied, although the total number in the individual groups is small. The percentage of eliminees with sickle cell anemia in the primary and basic groups is less than in those not showing the sickling trait. This may not be significant but does indicate that those with sickle cell anemia were at least maintaining a representative number in the various stages of training.

During the primary phase of training no high altitude missions were accomplished. In advanced training, however, numerous high altitude missions were flown; the percentage of sickling was comparable with the total number of cases of sickling found and to the general incidence of this condition. The four combat returnees with the sickle trait had flown both high and low altitude missions with the use of oxygen as is required of all pilots. The erythrocyte count, hemoglobin, reticulocyte count, and urine urobilinogen elimination were within normal level. The thin blood film revealed no typical sickled cells. There had been no unusual effects experienced while on high altitude missions. Total missions of these men ranged from 57 to 110 per person.

None of the twenty-three men positive for sickling had the drepanocytanemic or eunuchoid habitus described by others^{7, 8}; they were classified as physically fit. There was no history of anemia, unusual or prolonged abdominal pains, joint pains, resistant leg ulcers, or jaundice. Erythrocyte counts performed on fifteen of these men varied between 4,200,000 and 5,840,000. Hemoglobin content and white blood counts were within the normal range.

Effect of Lowered Oxygen Tension Induced in the Low Pressure Chamber on the Blood of Persons With Sickle Cell Anemia and the Sickle Cell Trait.—Four subjects with sicklemlia (Cases 1 to 4) and four controls (Cases 5 to 8) were utilized in this study. Four of the patients (Cases 1, 2, 5, and 6) had one previous experience in the low pressure chamber. They ranged in age between 20 and 24 years. One patient with active sickle cell anemia, a civilian resident of the near-by township, also volunteered for a simulated flight in the chamber. This subject had recently been discharged from the army because of anemia. He had a history of numerous acute episodes of joint pain, jaundice, and dyspnea on effort. At the time of examination he complained only of mild joint pains. He was able to perform his duties as an attendant at a near-by hospital without dyspnea. Physical examination revealed a canary-yellow color of the sclerae, apical systolic murmur, healed ulcers of the leg, and a drepanocytanemic habitus. The diagnostic parameter was 30.⁹ Previous erythrocyte counts in our laboratory varied between 2.7 and 3.2 million with reticulocyte counts of 6.2 and 7.5 per cent. There was 8 per cent sickling of erythrocytes on the Wright's stained film; from 20 to 25 nucleated red blood cells were found per 1,000 erythrocytes. The packed cell volume was from 30 to 32 mm. The white count varied between 12,000 and 14,000 with 6 per cent eosinophiles.

Physical examination and hematologic studies of the subjects with sicklemlia and controls were within the range of normal.

It was felt that total time of the simulated flight and the height reached would be dependent upon the physical capacities of the tested personnel to withstand the lowered oxygen tension. It was believed that it would be necessary to shorten the test when observations were made on the subject with active sickle cell anemia. Theoretically he was in a state of chronic oxygen want and the total oxygen content was lowered. Likewise, there was a probability that increased sickling might result in intravascular hemolysis and thrombosis. Before subjects entered the chamber, the following studies were made: erythrocyte count, hemoglobin determination, reticulocyte count, thin blood films stained

with Wright's stain, wet preparations for sickling, urinalyses for urobilinogen, icteric index, pulse rate, blood pressure, and activity of reflexes. Three subjects (Cases 2, 4, and 9) had electrocardiographic studies immediately before flight; in addition, two (Cases 4 and 9) had roentgen-ray examinations of the heart. All examinations listed were repeated after the descent. During the simulated flight reticulocyte preparations, dry thin blood films, and wet preparations were obtained at various levels. Thin blood films were reported positive for sickling, if the erythrocyte assumed the typical shape of the sickled cell. Close observation of the subjects for mental changes, visual disturbances, blood pressure changes, pulse rate, and reflex activity were also made. Oxygen was administered at the request of the individual subject or when, in the opinion of the examiners, it was thought necessary.

Oxygen arterial saturation levels were obtained on Cases 4 and 9 by means of the oxygen tensiometer which is a relatively inexact method for determination of the percentage of arterial oxygen saturation.

Forty-eight hours after completion of the test, erythrocyte counts, urine urobilinogen levels, and icteric indices were repeated on the subjects with sickle cell anemia and active sickle cell anemia. It is recognized that the icteric index and the amount of urobilinogen excretion in the urine do not provide accurate methods for estimating the degree of hemolysis, but facilities were not available for fecal urobilinogen determinations or plasma hemoglobin measurement.

RESULTS

The results of our studies are presented in Table II. All subjects except three (Cases 4, 5, and 9) were administered oxygen at 15,000 feet. Two subjects (Cases 4 and 9) were taken to 16,000 feet where after five minutes it was considered necessary to administer oxygen to one (Case 4). The other (Case 9), the subject with active sickle cell anemia, contrary to expectation, revealed less clinical evidence of oxygen want than any of the subjects. His ability to read the type I Jaeger chart at 15,000 feet surpassed that of the others. He was more relaxed and appeared fairly comfortable at the higher levels. Another subject (Case 5), an excitable and apprehensive person, needed oxygen at 10,000 feet. Two individuals (Cases 3 and 6) developed pronounced extrasystoles at the 15,000 foot level; these extrasystoles disappeared on descent. One subject (Case 9) was given oxygen in amounts comparable to that at ground level for a period of ten minutes after five minutes without oxygen at 16,000 feet.

In Table II, the results reveal no evidence of increased blood destruction. The erythrocyte count was frequently but not consistently increased after descent, but the reticulocyte count showed no change. Signs and symptoms of oxygen deficiency were not unusual for the subjects with sickle cell anemia and tended to follow the general pattern of our controls and other healthy subjects in the Army under diminished oxygen tension. A gradual increase in pulse rate, blood pressure, and activity of reflexes usually occurred and at 15,000 feet vision was sufficiently blurred to cause difficulty in reading the type I Jaeger chart.

TABLE II. RESULTS OBTAINED BEFORE, DURING, AND AFTER SIMULATED FLIGHT IN LOW PRESSURE CHAMBER

CASE	BEFORE SIMULATED FLIGHT										5000 FT. 15 MIN.				10,000 FT., 50 MIN. (BAR. PRESS. 522.6 MM. Hg)				15,000 FT., 30 MIN.				16,000 FT., 5 MIN. (BAR. PRESS. 411.8 MM. Hg)				AFTER SIMULATED FLIGHT				48 HOURS AFTER FLIGHT			
	ERYTHROCYTES (PER C.M.M.)	HEMOGLOBIN (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ART. OXYGEN SATURATION	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	HEMOGLOBIN (PER CENT)	RETICULOCYTES (PER CENT)	SICKLING (PER CENT)	ERYTHROCYTES (PER C.M.M.)	HEMOGLOBIN (PER CENT)	SICKLING (PER CENT)	ERYTHROCYTES (PER C.M.M.)	HEMOGLOBIN (PER CENT)	SICKLING (PER CENT)				
1st sicklemaia	5.2	101	N	96	0.0	N	98	0.0	0.0	98	0.0	N	98	0.0	N	98	0.0	0.0	98	0.0	0.0	101	0.0	N	5.0	110	N	0.1	5.4	110	N	0.1		
2d	4.6	88	N	96	0.0	N	96	0.0	0.0	98	0.0	N	98	0.0	N	98	0.0	0.0	98	0.0	0.0	88	0.0	N	4.6	86	N	0.3	4.8	86	N	0.3		
3d	4.3	85	N	96	0.0	N	96	0.0	0.0	98	0.0	N	98	0.0	N	98	0.0	0.0	98	0.0	0.0	85	0.0	N	4.3	83	N	0.3	4.5	83	N	0.3		
4th	4.8	95	N	96	0.0	N	96	0.0	0.0	98	0.0	N	98	0.0	N	98	0.0	0.0	98	0.0	0.0	95	0.0	N	4.8	93	N	0.4	5.0	93	N	0.4		
5th	5.1	90	N	96	0.0	N	96	0.0	0.0	98	0.0	N	98	0.0	N	98	0.0	0.0	98	0.0	0.0	90	0.0	N	5.1	86	N	0.2	4.8	86	N	0.2		
6th	5.4	110	N	96	0.1	N	96	0.1	0.1	98	0.1	N	98	0.1	N	98	0.1	0.1	98	0.1	0.1	110	0.1	N	5.7	110	N	0.3	5.7	110	N	0.3		
7th	4.3	90	N	96	0.2	N	96	0.2	0.2	98	0.2	N	98	0.2	N	98	0.2	0.2	98	0.2	0.2	90	0.2	N	4.8	88	N	0.3	4.8	88	N	0.3		
8th	4.9	90	N	96	0.6	N	96	0.6	0.6	98	0.6	N	98	0.6	N	98	0.6	0.6	98	0.6	0.6	90	0.6	N	5.2	88	N	0.8	5.2	88	N	0.8		
9th active sickle anemia	3.2	50	S	96	6.4	20	96	6.4	20	98	6.4	S	96	6.4	S	96	6.4	20	96	6.4	20	50	21	Pos.	3.6	60	S	0.21	3.9	60	S	0.21		
																																1/40		

Total time of simulated flight, two hours, eleven minutes.

Per cent sickling read on thin blood smear.

N, Not significant.

There was a moderate increase in the percentage of sickling as observed by the Wright's stained thin film taken at various levels and degrees of lowering of the oxygen saturation in Case 9. After ten minutes of oxygen at 15,000 feet a decrease in the degree of sickling was noted. These findings are demonstrated in Table II and Fig. 1. During the descent no oxygen was administered to this subject. His increase in erythrocytes was greater than the others and follow-up studies showed no evidence of any marked increase of hemolysis. Two months later his erythrocyte count was 3.6 million, and 8 per cent sickling on

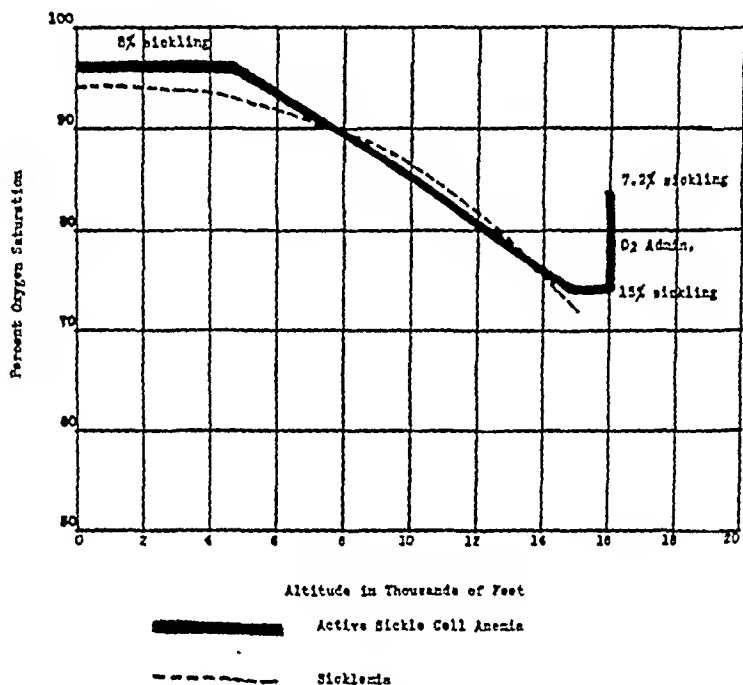


Fig. 1.—Percentage of oxygen saturation of blood at various altitudes up to 15,000 feet in a case of sickle cell anemia and 16,000 feet in a subject with active sickle cell anemia as determined by the oxygen tensiometer. Active sickle cell anemia subject given oxygen (approximately 40 per cent) ten minutes at 16,000 feet. The sickle cell anemia revealed no immediate sickling.

the Wright's stain. Examination of the wet and dry preparations taken immediately on the other subjects revealed no change. After twenty-four hours wet preparations obtained from the subjects with sickle cell anemia were positive for sickling.

Electrocardiographic and roentgenologic studies made before the test revealed no significant change after completion of the test.

COMMENT

From our observations the incidence of sickle cell anemia in the various training groups of Negroes at the Tuskegee Army Airfield is consistent with the reported incidence of this abnormality. Although the number of cases studied was small, it appears that the sickling trait played no significant part in the elimination of Negro cadets for flying deficiencies.

Wintrobe¹⁰ states that there is no evidence of definite geographic or racial differences at sea level in regard to the amount of hemoglobin or the number of erythrocytes in the circulating blood. Hurtado and associates¹¹ have presented an excellent study on the influence of anoxemia on the hemopoietic system. They believe that, in states of temporary anoxemia similar to our studies, any polycythemia noted is probably the result of a release of stored red blood cells and hemoconcentration; that corresponding to a repeated or constant exposure to a low pressure environment is related to an erythropoietic hyperactivity. In temporary anoxemia there are individual responses, and even a decrease in erythrocytes may occur.

Our subjects who volunteered for a simulated flight in the low pressure chamber were all healthy physically fit young males, with the exception of the man with active sickle cell anemia who could be classified as being in a state of chronic oxygen want. Our observations were primarily directed toward the detection of any evidence of massive erythrocyte destruction as a result of a lowering of the arterial oxygen tension to the levels of human intolerance in persons with the sickling trait. No sickling was observed immediately in any of the subjects with sicklelmia and increased erythrocyte destruction was not detected by our methods of testing. The subjects with sicklelmia varied in their response to high altitude as did the controls. Physical responses to lowered oxygen tension were not remarkable.

The results observed in the subject with active sickle cell anemia deserve further study and indicate that there is a gradual increase in sickling proportional to the decrease in the oxygen tension. However, an adequately sensitive compensatory mechanism resulted in an increase in erythrocytes which sufficed apparently for his oxygen needs. This increase was evidently the result of a release of stored red blood cells. It would appear that, although this subject was already in a state of oxygen want, he was able to respond even better to a diminished oxygen tension than did the controls or subjects with sicklelmia. There was no evidence of massive erythrocyte destruction in this subject, although, as previously stated, it is recognized that determination of urobilinogen excretion in the urine and the icteric index do not provide accurate methods for estimating rates of red blood cell hemolysis. However, an erythrocyte and reticulocyte count obtained simultaneously with these two tests should reveal the presence of massive hemolysis fairly accurately.¹²

It is unfortunate that more accurate methods could not be used in this study. The precautions outlined by Sherman⁶ in obtaining blood from the patient with sickle cell anemia would have presented much better evidence of increased intravascular sickling, since the role of atmospheric pressure in causing this phenomenon cannot be excluded.

However, the decrease in sickling noted after administration of oxygen minimizes to some extent the effect of atmospheric conditions on the erythrocytes in vitro and is compatible with the studies of Reinhard and associates¹³ who administered high oxygen concentrations to subjects with sickle cell anemia and obtained a decrease in the degree of sickling.

SUMMARY AND CONCLUSIONS

In a study of the incidence of sickle cell anemia among Negro cadets in various stages of training and combat returnees, we found an incidence of 7.37 per cent which compares favorably with the over-all percentage of sickling in the Negro race. There was no evidence of an increased elimination of cadets because of the sickling trait.

Under lowered arterial oxygen tension near the levels of human intolerance, none of the subjects with sickle cell anemia who were tested revealed evidences of immediate intravascular sickling or increased erythrocyte hemolysis.

A volunteer subject with active sickle cell anemia withstood the lowered oxygen tension even better than did the individuals with sickle cell anemia or the control subjects, although there was suggestive evidence of increased intravascular sickling. Administration of oxygen for a short period resulted in more normal erythrocytes. This suggests that adequate administration of oxygen will protect patients who have active sickle cell anemia under conditions which ordinarily will result in a lowered oxygen tension and increased intravascular sickling. Even without oxygen therapy there was no evidence of massive erythrocyte destruction.

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THE BONE MARROW IN HEMOPHILIA DURING LIFE^c

A STUDY OF THE CELLULAR ELEMENTS AND THE COAGULATION TIME OF THE MARROW BLOOD

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THE bone marrow in hemophilia during life has not been studied. In fact, hemophilia is the only condition listed in which sternal puncture is contra-indicated.^a Bone marrow studies in this disorder have been limited to autopsy material. Cnster and Krumbhaar¹ reviewed the literature and studied in detail the histopathology of the hemopoietic tissues in three patients with hemophilia who died in various phases of the hemorrhagic disorder. As hemophilia is characterized by a prolongation of the coagulation time of blood, the megakaryocytes of the bone marrow were of particular interest. The coagulation time of the marrow fluid was determined and compared with the coagulation time of the peripheral blood.

METHODS

The method of sternal aspiration and preparation of the marrow specimens has been reported in detail in a previous paper.³ Under aseptic technique and procaine anesthesia the sternum is punctured in the second or third interspace with a specially devised 16 gauge needle. The needle is forced into the bone perpendicular to the sternum until a sudden "give" indicates that the marrow cavity has been reached. The stylet is then removed. With a tightly fitting, dry 5 c.c. syringe, about 1 c.c. of material is aspirated and immediately placed in a paraffin-lined tube containing a minute amount of heparin. Without delay a second dry syringe (10 c.c. size) is attached to the sternal needle, and from 3 to 5 c.c. of marrow fluid are aspirated. This "marrow blood" is practically devoid of hemopoietic elements, since most of the marrow elements are removed in the first 1 c.c.; it is expelled carefully into a clean dry sterile serologic tube 21 mm. in diameter, care being taken to prevent the formation of air bubbles. The tube is tilted every one or two minutes, and when the tube can be inverted without the blood flowing out, coagulation is considered complete.

The 1 c.c. of heparinized material consisting of sinusoidal blood, hemopoietic marrow, and fat is pipetted into a hematocrit tube and centrifuged from 2,000 to 2,500 r.p.m. for five minutes. Centrifugation separates the following layers reading from top down in the tube: (1) fat, (2) plasma, (3) myeloid-erythroid cells (this layer also contains the megakaryocytes), and (4) erythrocytes (sinusoidal blood). The heights of the several layers are recorded. The fat and plasma are removed separately and discarded. The myeloid-erythroid layer, together with a small amount of the erythrocyte layer, is pipetted off separately, transferred to a paraffined watch glass, mixed, and from this material films are made and stained with Wright's stain and studied microscopically for cell distribution and types.

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Immediately preceding the marrow aspiration, 10 c.c. of peripheral blood are withdrawn by venipuncture into a dry, sterile syringe. Five cubic centimeters of the blood are expelled carefully into a clean, dry, sterile serologic tube 21 mm. in diameter, and the coagulation time is determined (Howell's method). The remaining 5 c.c. of blood are transferred to a bottle containing a measured amount of ammonium and potassium oxalate mixture as an anticoagulant. This blood is shaken thoroughly and used immediately for the following studies: hemoglobin determination, erythrocyte and leucocyte counts, differential counts, and reticulocyte and platelet estimations. The volume of packed cells, sedimentation rate, and icterus index are determined by Wintrobe's method.¹¹ Mean corpuscular volume, hemoglobin, and hemoglobin concentration are then computed.

When the stained films of the marrow are studied, the ratio of the myeloid to the erythroid cells, the dispersion of the nucleated red cells, the dispersion of the myeloid series, and a differential count of the megakaryocytes designated as young, adult, and degenerated are recorded. Under young forms are included the promegakaryocytes, the earliest type of megakaryocyte, seen chiefly in essential thrombocytic purpura and the intermediate forms seen usually in chronic hemorrhage.

In recording the erythroid series the terms pronormoblast, basophilic, polychromatophilic, and orthochromatic (acidophilic) normoblasts are used in order of their increasing maturity. Similarly for the myeloid series the terms myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and polymorphonuclear are used. Plasma cells, tissue cells, lymphocytes, and monocytes are noted when present but are not included in the counts used to determine the foregoing percentage relationships.

Other laboratory determinations such as clot retraction and bleeding time, tourniquet test, plasma ascorbic acid, and prothrombin time were performed.

RESULTS

Four patients with hemophilia were studied during various stages of activity. All four subjects had typical hereditary and individual histories of bleeding episodes and had been followed for a number of years in the pediatric, orthopedic, and medical wards.² The two younger boys had hemophilic joints; the Negro with hemophilia had a leg operation at which time the hemophilic condition was discovered; and the fourth patient, a 51-year-old man, had a carcinoma of the penis. In Table I the coagulation times of the peripheral blood and bone marrow blood are compared with similar studies in a variety of hematologic disorders and other clinical conditions. In two patients with hemophilia studied during the remission phase, the blood studies and the coagulation time (Howell's method) of the peripheral blood were 20 minutes and 25 minutes, respectively, while the coagulation of the bone marrow blood was 25 minutes and 29 minutes, respectively. These values are normal and within the range of technical error. In the two subjects with hemophilia studied during an exacerbation of the hemorrhagic disorder, with a macrocytic anemia in one subject and a normocytic anemia in the other, the coagulation time of the blood was 90 minutes and 150 minutes, respectively, while the marrow blood coagulation time was 150 minutes in both subjects.

In normal controls, patients with polycythemia vera, normocytic, microcytic hypochromic (due to hemorrhage), macrocytic anemias, leucemias, and thrombocytopenic purpura, the coagulation time of the marrow blood varied between 15 seconds and 3 minutes while that of the peripheral blood taken

TABLE I. LABORATORY FINDINGS IN BLOOD AND BONE MARROW IN PATIENTS WITH HEMOPHILIA AND VARIOUS CLINICAL DISORDERS

DISEASE	BLOOD							BONE MARROW	
	AGE AND RACE	BLEEDING TIME— DUKE METHOD (MINUTES)	COAGULATION TIME— HOWELL METHOD (MINUTES)	CLOT RETRACTION TIME (HOURS)	CEMENTIC ACID	PROTHROMBIN TIME	TOURNIQUET TEST	COAGULATION TIME— HOWELL METHOD (MINUTES)	CLOT RETRACTION (HOURS)
Hemophilia T.S.	51 W.	3.0	20	6	Normal	Normal	Neg.	25	1½
Hemophilia I.H.	20 C.	3.5	25	2½	Normal	Normal	Neg.	29	3
Hemophilia H.B.	12 W.	4.0	190	1	Normal	Normal	Neg.	150	6
Hemophilia T.B.	3 W.	5.5	150	18	Normal	Normal	Neg.	150	4
Normals (4)*			16					2.5	
Pernicious anemia (2)			15					2.15	
Normocytic anemia (1)			15					2	
Hemorrhagic anemia (2)			14					1.30	
Refractory anemia (1)			5					3	
Leucemia (1)			12					2	
Thromboeytopenic purpura (1)			10					3	
Polycythemia vera (2)			7					2.15	
Cirrhosis of liver with jaundice (1)			9					3.30	
Infectious mono- nucleosis			6.30					13	
Pulmonary tuberculosis (1)			4.25					3	

*Number of patients.

from the arm vein ranged from 5 to 18 minutes. In a subject with pulmonary tuberculosis the relationship of the coagulation time of the marrow blood to that of the peripheral blood was from 3 to 4 minutes, 25 seconds, and in a subject with infectious mononucleosis the marrow blood coagulated in 13 minutes while the peripheral blood clotted in 6 minutes, 30 seconds.

The peripheral blood (Table II) in the two patients with hemophilia revealed a macrocytic and hypochromic anemia in one patient and a normocytic anemia in another; in neither patient was the anemia severe. In both of these patients there was a slight polymorphonuclear leucocytosis and a normal to increased number of blood platelets. The other blood studies showed no significant change from normal.

The bone marrow (Table II) in two subjects with hemophilia, studied during the remission phase with normal blood findings and coagulation time, revealed a normal quantitative and qualitative erythroid and myeloid pattern. The megakaryocytes were quantitatively and morphologically normal. There

TABLE II. BLOOD AND BONE MARROW FINDINGS IN PATIENTS WITH HEMOPHILIA AND IN OTHER CLINICAL CONDITIONS—CONT'D

PATIENT, AGE, AND RACE	MYELOID ERYTHROID (VOL. %)	FAT VOLUME (%)	ERYTHROID (PER CENT)				MYELOID (PER CENT)										MYELOID-ERYTHROID RATIO (%)			MYEUKARYOCYTES (PER CENT)		
			PROERYTHROBLASTS	BASOPHILIC NORMOBLASTS	POLYCHROMATIC NORMOBLASTS	ORTHOCHROMATIC NORMOBLASTS	MYELOBLASTS	PROMYELOCYTES	NEUTROPHILIC MYELOCYTES	NEUTROPHILIC MYELOCYTES	NEUTROPHILIC METAMYELOCYTES	EOSINOPHILIC METAMYELOCYTES	POLYMONONUCLEAR NEUTROPHILES	EOSINOPHILES	BASOPHILES					IMMATURE	MATURE	DEGENERATED AND FREE NUCLEI
T. S., 51, W.	8.0	2.0	3.0	12.0	85.0	0.0	0.0	2.0	12.0	7.0	56.0†	3.0	13.0	1.0	1.0	07/23	11.0	61.0	28.0			
Patients with hemophilia																						
T. H., 20, C.	6.0	2.0	1.0	8.0	91.0	0.0	0.0	2.0	30.0	1.0	19.0	1.0	15.0	2.0	0.0	63/37	5.0	75.0	20.0			
H. B., 12, W.	12.0	1.0	7.0	15.0	75.0	3.0	0.0	0.0	15.0	6.0	60.0	2.0	15.0	1.0	1.0	57/43	10.0	64.0	26.0			
T. W., 12, W.	14.0	2.0	9.0	25.0	58.0	8.0	0.0	1.0	11.0	1.0	78.0	3.0	10.0	1.0	1.0	46/54 2.75:	7.0	57.0	36.0			
T. B., 13, W.	6.8	3.2	2.6	13.2	79.3	5.9	0.4	0.9	7.8	3.0	56.8	5.0	23.1	2.5	0.5	1.0	24.0	18.0	28.0			
Normals (10)* 13, W.																	14.4	33.8	21.8			
Essential thrombopenic purpura (5)	18.0																52.0	24.0	24.0			
Chronic thrombopenic purpura (1)	7.0																34.0	33.0	33.0			
Secondary thrombopenic purpura (1)	30.0																31.5	42.0	26.5			
Symptomatic thrombopenic purpura (3)	11.0																31.0	48.0	21.0			
Chronic hemor- rhagic (5)	11.8																11.0	75.0	14.0			
Acute hemor- rhagic (3)	12.0																					

*Number of patients.
†Includes band forms.

were large numbers of normal appearing platelets and many free megakaryocyte nuclei. The mature types of megakaryocytes were increased in number. The two patients with hemophilia who were studied during a bleeding episode revealed a hyperplastic bone marrow (myeloid-erythroid volume). The mature types of megakaryocytes were increased and, as in the first two subjects with hemophilia, there were large numbers of formed platelets and free megakaryocyte nuclei.

COMMENT

In the reported necropsies on hemophilia either the bone marrow was not examined or little of histologic significance concerning the marrow picture was reported.¹ Ricker² stated that the vertebral and femoral marrows were not hyperplastic. Custer and Krumbhaar,¹ who were the first to make a comprehensive histologic study of the bone marrow as well as of the other hemopoietic tissues, remarked that the histopathology of the hemopoietic tissues in hemophilia was an unexplored field. In the three patients reported on by Custer and Krumbhaar the blood forming tissues all showed normal regenerative ability, the first two were predominately erythroblastic in character and the third showed a leucoblastic type of regeneration. All three patients showed a marked increase of early types of the thrombocytic series (megakaryoblasts) and megakaryocytes. The morphology of the megakaryocytes did not vary from the normal, and Wright's figures (pseudopods of the megakaryocyte cytoplasm) were occasionally observed. Autopsies were performed on Custer and Krumbhaar's patients following hemorrhagic episodes.

In our four patients with hemophilia the bone marrow pattern (Table II) could be correlated with the stage of the hemorrhagic disease. During the bleeding phase of the disorder with a prolonged coagulation time, the bone marrow showed a uniform hyperplasia of all elements with varying degrees of myeloid and erythroid hyperplasia. The megakaryocytes were increased in number but were morphologically normal. During the remission phase with normal blood findings and a normal coagulation time, the bone marrow revealed a normal erythroid and myeloid pattern, and the megakaryocytes were normal in number and morphology. In all the marrows the number of formed and morphologically normal platelets were increased with numerous free megakaryocyte nuclei. The augmented megakaryocyte nuclei may be due in part to the mechanical damage or rupture of the large megakaryocytic mass with extrusion and breaking up of the large multilobulated nucleus. This mechanical mechanism does not account for the large numbers of formed platelets. The predominant number of adult megakaryocytes which exceed the total of both immature and degenerated types of megakaryocytes is evidence of an accelerated rate of platelet formation (Table II). Many of the formed platelets in the marrow probably degenerate in situ in view of the fact that the peripheral blood does not reflect the increased plateletogenesis in the bone marrow. In normal control subjects⁴ and in patients with thrombocytopenic purpura⁵ (Table II), the combined number of immature and degenerated types of megakaryocytes exceeds the mature or adult types of megakaryocytes. It is

interesting to note that in acute thrombocytopenic purpura with hemorrhage there is a megakaryocytic hyperplasia of the young forms (immature), and in the less acute phase or chronic stage the mature types appear; however, the platelets are markedly decreased or absent in both the bone marrow and peripheral blood. This is due to faulty maturation and a lack of platelet formation of the megakaryocytes in the bone marrow.⁶

The origin, formation, and morphologic description of the megakaryocytes in human adult bone marrow under normal and pathologic conditions has been discussed and illustrated elsewhere.⁶ Mature megakaryocytes are thought to be formed from the myeloblast through a process of heteroplastic hemopoiesis in contrast to the homoplastic hemopoietic development of the myeloid¹⁰ and erythroid cells.⁷ If this concept is correct, any disturbance involving the stem cell (myeloblast) will eventually interfere with the normal development and platelet formation of the megakaryocyte in the bone marrow. The formation of platelets occurs by a process of budding or by detachment of portions of the cytoplasm (Wright's figure) of the megakaryocytes in the bone marrow. It occurs as a normal process and more frequently by cytolysis of the cytoplasm of these cells as observed in aspirated human bone marrow.⁶

The increased number of platelets and free megakaryocyte nuclei seen in the marrow of patients with hemophilia is similarly observed in the marrow following acute hemorrhage.

The coagulation time of the marrow blood in patients with hemophilia equaled or exceeded the coagulation time of the blood taken from the arm vein. In one subject the marrow coagulation time was one hour longer than that of the peripheral blood. On the other hand, in normal controls, blood disorders, and various clinical conditions the coagulation time of the peripheral blood was several times that of the marrow blood. The only exception was in a patient with infectious mononucleosis in which the marrow coagulation time exceeded that of the peripheral blood.

The cause of the prolonged coagulation time of the marrow blood in hemophilia and the accelerated rate of platelet formation in the bone marrow is not entirely clear. One may speculate that there exists some relationship which results in a type of platelet that is morphologically normal but physiologically abnormal (in other words, an immature type of platelet which is physiologically and abnormally resistant due to the accelerated rate of the plateletogenesis in the bone marrow), and this is reflected in the peripheral blood by increased stability of the platelets. The abnormal plateletogenesis in the marrow may be due to the abnormal environment (hemophilic marrow plasma) or may equally well be regarded as an abnormal environment (prolonged marrow coagulation) due to the abnormal plateletogenesis which results in the slow liberation of thromboplastin from the platelets.

CONCLUSIONS

1. In patients with hemophilia who have a coagulation time of 2 hours, 15 minutes, sternal aspiration is not a contraindication.

2. The megakaryocytes in the bone marrow in patients with hemophilia are morphologically normal.
3. The megakaryocytes in the bone marrow in subjects with hemophilia appear to have an accelerated rate of maturation and platelet formation when compared with the maturation rate and platelet formation seen in the bone marrow in various other clinical conditions.
4. The hyperplasia of the myeloid and erythroid elements can be definitely correlated with the hemorrhagic tendency of the disease.
5. The coagulation time of the marrow blood in patients with hemophilia exceeds the coagulation time of the peripheral blood in contrast to the reverse relationship observed in normal individuals and in various clinical conditions.
6. The abnormal rate of plateletogenesis in the bone marrow and the increased coagulation time of the marrow blood may be associated with the pathologic physiology of hemophilia.

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AN UNUSUAL CASE OF GENERALIZED ANASARCA APPEARING IN AN IMPOUNDED DOG WITH CIRRHOSIS OF THE LIVER

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WATER AND ELECTROLYTE DISTRIBUTION IN BLOOD AND TISSUE

THIS report of a case of generalized anasarca of unknown origin in an impounded dog is of special interest not only because of the anasarca but because of the presence of an extensive ascites. The purpose of this paper, therefore, is to present data on the water and electrolyte distribution in blood and tissues of an animal with an extensive gross edema of the entire body. The data obtained should indicate possibly a maximum disturbance in the equilibria of water and electrolytes of the body under conditions of gross edema and ascites.

A Boston bull terrier, male, weighing approximately 12 kilograms, was received from the pound (Fig. 1). He was unable to stand because of the extreme gross edema of the hind- and forelegs which had transformed the extremities into useless trunks because of their weight. The presence of a large volume of ascitic fluid further hindered his power to stand. Extreme pitting, soft edema was distributed throughout the body; even the face was swollen. The skin of the legs and abdominal wall oozed under the least pressure produced by slight rubbing. The subcutaneous fluid could be shifted around with a change in the position of the animal. The dog was therefore anesthetized and operated upon.

Since the subcutaneous fluid and edema obscured the femoral vein for nembutal injection, the region was cocaineized, an incision then made, and the vein exposed for injection of the anesthetic. Blood was taken from the exposed femoral artery; 20 c.c. were withdrawn under oil for the serum analyses, and 10 c.c. were defibrinated for whole blood analyses. After blood and tissue had been removed, the dog was killed.

Autopsy showed that the dog had not eaten lately, for the stomach and the pale edematous gut were empty. The bladder also was empty. The peritoneal cavity contained two liters of ascitic fluid; fluid was present in the pleural cavity. The heart and kidneys seemed normal in size. There was no endocarditis. The liver was enlarged and cirrhotic; adhesions to the surrounding viscera were not present. There was an increased firmness and a gross nodularity of the tawny brown surface.

The microscopic changes* are summarized as follows.

Liver.—The sections of the liver showed large areas of atrophy and disappearance of liver cells with the blood sinuses forming alveolar blood spaces. These areas ran across many lobules, usually sparing the periportal region and not definitely related to the central veins. Around these areas were masses of pigment-filled cells with some leucocytic infiltration. The

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*Kindly done by Dr. Louis Leiter.

portal areas in this part of the liver showed distention of bile ducts with mucus, degeneration of the epithelium, and considerable infiltration with inflammatory cells. In the areas of hemorrhagic necrosis there was marked increase in connective tissue. The whole picture suggested an infection of the bile ducts with some obstruction, secondary fibrosis, and a considerable destruction of liver parenchyma which eventually would have ended in a severe biliary cirrhosis.

Kidneys.—The sections showed relatively large glomeruli with thick framework or basement membrane with moderate increase in fibrous tissue in some but no evidence of chronic inflammation or acute change. There were no large scars and no protein in the glomerular spaces. The tubules showed considerable degeneration with protein and colloid droplets in the lumen of the convoluted tubules and a few casts in the medullary region. No calcification and no interstitial inflammation nor remarkable changes were seen in the blood vessels. There was no evidence of prolonged tubular disease with atrophy and secondary fibrosis. All in all, the kidneys might be considered normal for an old dog, except for the tubular degeneration which could be a terminal event.

Heart.—The sections showed no interstitial edema, no evidence of inflammation, no remarkable increase in connective tissue, and no vascular changes.

Skin.—The sections showed a massive edema of the entire thickness with swelling of the collagen bundles of the corium and new capillary formation in the loose subepidermal zone and also in the deeper layers of the skin. There was no evidence of inflammation or infection. The small arteries showed thick fibrous media which might indicate the age of the dog.

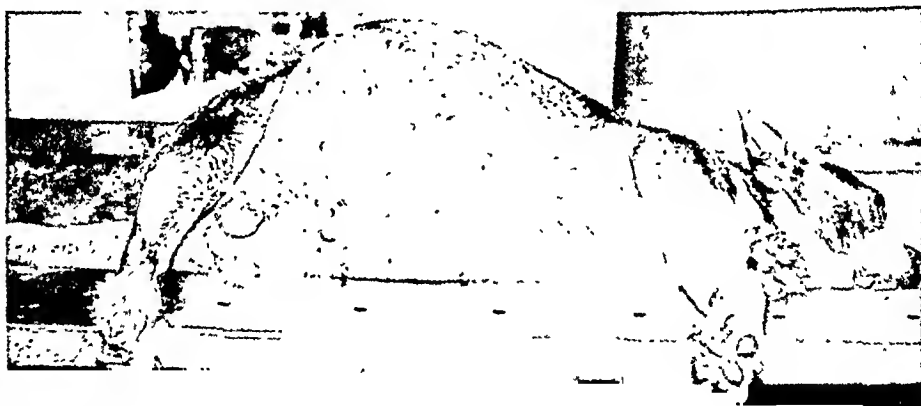


Fig. 1.—Impounded dog showing ascites and generalized anasarca.

PROCEDURES AND METHODS

The physiologic procedures and chemical methods used here were the same as those employed in previous studies on blood,^{1, 2} skeletal muscle,^{3, 4} skin,⁴ and brain.⁵ The following determinations were made: on the ascitic fluid, water, chloride, sodium, potassium, calcium, magnesium, total nitrogen, and NPN; on the serum, water, chloride, sodium, potassium, calcium, magnesium, NPN, total protein, and A/G ratio; on the whole blood, water, chloride, sodium, potassium, and cell volume; on the skin, fat, water, chloride, sodium, potassium, calcium, magnesium, and total nitrogen; on skeletal muscle, fat, water, chloride, sodium, potassium, calcium, magnesium, and content of blood; on the cerebellum and cerebral hemispheres, water, chloride, sodium, potassium, and total nitrogen.

All ascitic fluid, serum, and blood analyses were made in duplicate. The composition of the red blood cells was estimated from the analyses of the whole blood and serum and the accompanying measurement of cell volume. All tissues analyses were made either in duplicate or quadruplicate.

The analytic data on muscle were subjected to the same method of calculation as that used previously² to estimate phase volumes of skeletal muscle. Briefly,
$$(F) = \frac{(Cl)_M \times (H_2O)_S \times 1000}{1.04 \times (Cl)_S}$$
 in which (F) represents the amount of extracellular phase per kilogram of muscle, and the subscripts M and S represent muscle and serum, respectively. From the value for (F) the intracellular phase (C) of 1 kg. of muscle was estimated: $(C) = 100 - (F)$. From the value for (C) the intracellular water $(H_2O)_C$ was estimated: $(H_2O)_C = (C) - (S)$, in which S represents solids per kilogram of muscle.

The tissues were removed from the animal as follows: The chest and abdomen were shaved, and the skin was washed with distilled water and dried with gauze. A section of skin approximately 8 by 10 cm. was dissected from the ventral wall of the abdomen and chest and placed in a glass stoppered weighing bottle. One of the rectus abdominis muscles was then removed. Although these tissues were handled with the greatest of care, some fluid was lost from them as they were being removed and quickly placed in glass stoppered weighing bottles. The removed skin and skeletal muscle were immediately placed on a tile and trimmed quickly to remove all visible connective tissue, blood vessels, and fat. The muscle was returned to the weighing bottle and minced with scissors, and aliquot parts were weighed for all analyses. The skin was cut into small strips approximately from 1.5 to 2 mm. and placed in a weighed, glass-stoppered weighing bottle and, after being weighed, was placed in an oven at 102° C. and dried to a constant weight. The dried skin was then extracted for neutral fat, after which the strips of skin were transferred quantitatively to a special apparatus and pulverized. The pulverized mixture was returned to a glass stoppered weighing bottle, and aliquots were weighed for all analyses.

The brain was removed after the muscle and most of the bone had been taken from the skull. The brain stem and cerebellum, after separation by section through the peduncles at the level of the superior colliculi, and also the cerebral hemispheres were placed in glass stoppered weighing bottles. The tissues were minced with scissors, and weighed aliquots were used for all analyses.

RESULTS

Analytical Data on Blood and Tissues

Values for Constituents of Serum, Ascitic Fluid, and Blood Cells.—In Table I are presented the analyses of the serum, ascitic fluid, and blood cells from the dog with generalized anasarca and from normal dogs, the normal averages being included for comparison. When the findings are compared, the greatest differences are found in the water, protein, potassium, calcium, and

TABLE I. ANALYSES OF ASCITIC FLUID, BLOOD SERUM, AND CELLS FROM NORMAL DOGS AND DOG WITH ANASARCA

	H ₂ O (GM.)	Cl (MEQ.)	Na (MEQ.)	K (MEQ.)	Ca (MEQ.)	Mg (MEQ.)	NPN (MG.)	TOTAL PRO- TEIN (GM.)	CELL VOLUME (C.C. PER 100 C.C.)	ALBU- MIN (GM.)
Normal dogs*										
Serum	922.0	107.1	141.2	3.95	4.94	1.73	285.0	58.5		
at	5.1	2.3	3.5	0.40	0.14	0.24				
Cells	703.0	62.3	93.9	9.37			22.0	3.1		
at		6.4	7.7	1.1					46.8	
									5.4	
Anasarca dog										
Serum	39.6	109.7	140.2	5.70	4.06	5.24	758.0	42.2		
Cells	744.0	64.8	91.4	6.89						27.2
Ascitic fluid	956.3	112.5	138.8	5.88	3.60	1.80	662.0	12.0	36.0	

The concentrations are expressed per kilogram of fluid and serum and per liter of red cells.

*See Reference 6.

†Standard deviation.

magnesium content of the serum and the water content of the cells. The water content of 93.96 per cent in the serum was the result of an osmotic adjustment following the lowering of the protein concentration to 4.22 per cent. The lowered serum calcium value of 4.0 meq. per kilogram occurred in response to the lowered protein concentrations in order to maintain a normal calcium ion concentration. The large increase in magnesium is spectacular. It may reflect soft tissue breakdown and renal excretory impairment.

There was a moderately low hematocrit value of 36 per cent, which might be explained as a result of existing hemodilution.

The ascitic fluid showed a protein content of 1.2 Gm. per cent. This value for protein content indicates that there has been little alteration in the permeability of the capillaries or else a considerable protein content would have been obtained. Since the ascitic fluid usually shows more protein than does the subcutaneous edema, the general edema fluids must have contained less than one per cent of protein.

TABLE II. ANALYSES OF TISSUES FROM NORMAL DOGS AND DOG WITH ANASARCA

	H ₂ O (GM.)	Cl (MEQ.)	Na (MEQ.)	K (MEQ.)	Ca (MEQ.)	Mg (MEQ.)	TOTAL NITROGEN (GM.)
Normal dogs							
Muscle*	775.0	18.41	23.07	98.4	1.63	18.11	
α	8.6	3.6	4.20	7.5	0.35	1.87	
Skin†	708.3	86.7	96.5	22.46	3.01	3.03	46.8
α	20.1	2.5	4.2	2.7	0.52	0.37	3.7
Cerebellum‡	745.0	35.19	50.8	92.7	2.14	10.80	19.1
α	7.0	0.89	1.7	4.0	0.14	0.60	0.5
Hemisphere‡	761.3	36.71	51.0	95.6	2.14	11.26	18.9
α	8.3	1.05	2.4	4.7	0.14	1.12	0.3
Anasarca dog							
Muscle*	816.0	45.45	55.8	81.8	2.8	11.12	
Skin†	866.6	101.0	84.9	16.1	4.51	3.71	21.6
Cerebellum‡	742.2	38.1	56.6	88.4			19.3
Hemisphere‡	768.8	39.0	55.7	87.5			18.5

The concentrations are expressed in units per kilogram.

*Units are expressed per kilogram of fat-free, blood-free muscle.

†Units are expressed per kilogram of fat-free skin.

‡Units are expressed per kilogram of wet brain.

Values for Constituents in Tissues.—In Table II are presented the analyses of skin, skeletal muscle, and brain from the edematous animal and from normal dogs. In Table III are presented the values expressed in units per 100 Gm. solid. When the tissue findings from the edematous dog are compared with those from normal dogs, the greatest differences are found in the skin and muscle values. The brain tissues, cerebellum, and cerebral hemispheres demonstrated no change in total water content and only small increases in chloride and sodium concentrations. Relative increases are shown in the total water of 15.8 per cent in skin and 4.1 per cent in skeletal muscle; in the chloride of 17.3 meq. per kilogram of skin and 27.04 meq. per kilogram of muscle; and in the sodium of 32.7 meq. per kilogram of muscle. The potassium was 6.3 meq. lower in skeletal muscle than in the normal animals. These findings on skin and muscle therefore suggest an enormous extracellular phase volume accompanied by a lowered intracellular phase volume.

TABLE III. TISSUE ANALYSES OF NORMAL DOGS AND ANASARCA DOG

	H ₂ O (GM.)	Cl (MEQ.)	Na (MEQ.)	K (MEQ.)	Ca (MEQ.)	Mg (MEQ.)	TOTAL NITROGEN (GM.)
Skin							
Normal	243	29.7	33.1	7.71	2.06	2.08	16.0
Anasarca	652	78.0	63.6	12.08	1.70	1.40	16.2
Muscle							
Normal	344	8.2	10.2	43.8	0.73	8.05	
Anasarca	444	24.7	30.3	44.5	1.52	6.05	
Cerebellum							
Normal	292	13.8	19.9	36.3			7.5
Anasarca	288	14.8	21.0	34.3			7.5
Hemisphere							
Normal	328	15.7	21.8	40.9			8.1
Anasarca	333	16.0	24.2	37.9			8.0

All values are expressed in units per 100 Gm. solid.

DISCUSSION

Although it is almost impossible to decide the exact etiologic factors in this dog's edema, it is possible to make a few tentative speculations.

The total serum protein concentration of 4.22 Gm. per cent, of which 2.72 Gm. was albumin and 1.50 Gm. was globulin, could have been one of the causal factors. The serum proteins were reduced to a level where the colloid osmotic pressure of the plasma was not sufficient to counterbalance the hydrostatic pressure in the capillaries. This tends to force fluid out of the blood into the tissue spaces. Similar filtration types of edema with low serum protein content have been observed in nephrotic types of edema, in malnutrition, and in every disturbance of the formation of serum proteins.⁷

Whether the low protein values were the result of a loss of proteins in the urine or malnutrition or a deficient generation resulting from disease, or a combination of all these factors cannot be stated. Albuminuria, for which we have no direct evidence, could not have been responsible with the serum albumin still 2.72 Gm. per cent. Furthermore, the serum cholesterol content of 192 mg. per cent suggests the absence of tissue starvation.

The anatomic changes in the liver suggest that the severe liver disease may have been sufficient to have interfered with the formation of the serum proteins and so accounted for the hypoalbuminemia. Zeldis and Alling⁸ have given evidence to suggest a retarded rate of restoration of albumin in association with cholangitis and hepatitis in a dog.

It is interesting that the level of the serum albumin in this dog with cirrhosis of the liver and ascites is similar to those presented on many patients with cirrhosis of the liver. Post and Patek⁹ have reviewed the extensive literature and have suggested that although the reduction in serum albumin is the essential factor in the formation of ascites it also seems likely that hypertension of the portal vein must determine the site at which the transfer of fluid takes place.

It seems unlikely that the albumin value of 2.72 Gm. per cent in the serum of this animal is low enough to cause the massive edema. The blood protein changes could have been secondary to the loss of protein in the ascitic fluid and the edema fluid. Therefore, there must have been other factors in the dog's

edema. The possibility of heart failure with increase in venous pressure, aggravated in this case in the portal area by the changes in the liver, must be considered.

The histologic changes in the kidney do not indicate that the edema was the result of organic renal excretory insufficiency. Without a doubt the nonprotein nitrogen retention (76.8 mg. per cent) in the serum was masked to a large degree by the dilution of waste products in the edema fluid. In view of the relatively well-preserved kidneys one must assume prerenal or circulatory azotemia with oliguria. The latter would aggravate any underlying tendency to edema.

Although we have attempted to find the cause of the generalized anasarca in a vagrant, imponded dog, the purpose of the study was to present the data on the water, protein, and electrolyte distribution in blood and tissues. These data might be considered a maximum disturbance in the equilibria of water, protein, and electrolytes resulting from gross edema and ascites.

The view that the skin is a great reservoir capable of taking care of large volumes of water^{4, 10} is again demonstrated here. Skin under normal conditions contains an average of 70.83 per cent of water. In this dog there was rise to 86.66 per cent, which is actually 22.2 per cent higher than the normal skin value. The distribution of this water in skin cannot be definitely ascribed, but the tremendous increases in sodium and chloride concentrations indicate that the large increase is in the extracellular phase. In skeletal muscle the total water content was 81.60 per cent, or 4.1 Gm. of water higher than the muscle removed from normal dogs. This actually represents a 5.3 percentage increase in water. If the distribution of this water is computed (muscle, anasarca, Table IV), the extracellular phase (F) is 375 Gm. or a 150 per cent increase over the value for a kilogram of normal dog muscle. Some of this increase was the result of a transference of water from the intracellular phase, since the volume of intracellular water $(H_2O)_c$ per kilogram was estimated to be 441 Gm., the normal value being 620 Gm.

To estimate the absolute changes in volume which the normal muscle has undergone during the course of disease, we assume that the muscle before the

TABLE IV. PHASE VOLUME DATA ON MUSCLE FROM ANASARCA DOG

Muscle	M	(F)	$(H_2O)_c$	(S)	$(H_2O)_c$
Normal*	1000	155	620	225	733
Anasarca	1000	375	441	184	
(M) _t	1224	459	540	225	705
Δ	+224	+304	-80		-28

All values are expressed in grams per kilogram of fat-free, blood-free muscle.

M = Extracellular phase and intracellular phase.

(F) = Grams of extracellular phase per kilogram of muscle.

$(H_2O)_c$ = Grams of intracellular water per kilogram of muscle.

$(H_2O)_c$ = Grams of water per kilogram of muscle cells.

(S) = Solids of intracellular phase.

(M)_t = Absolute final weight of the initial kilogram of normal muscle after the effects of the disease.

Δ = Differences between values obtained on normal muscle and the absolute values following the effects of the disease (M)_t.

*It is assumed that the initial muscle is the normal muscle of the dog before the onset of disease.

onset of disease was a normal muscle possessing normal volume values (normal muscle, Table IV). Also, we assume that during the disease there has been no alteration in the amount of solids present in that quantity of muscle cells in a kilogram of normal muscle—(S) normal muscle, Table IV. In other words, if the cell solids of a kilogram of normal muscle (S)_N is divided by the cell solids of a kilogram of muscle as removed from the anasarca dog (S)_A, a factor is obtained to multiply all volume values of the anasarca muscle to obtain the absolute values—(M)_r, Table IV. The absolute values so obtained showed that an increase of 224 Gm. per kilogram of control muscle (M) had occurred, of which a 304 Gm. increase were attributed to the extracellular phase (F) and a loss of 80 Gm. (C) from the muscle cells. Actually, the extracellular phase volume of the original kilogram of muscle is now 459 Gm. Thus, in this animal the edema of the skeletal muscle is all extracellular and is accompanied by a dehydration or shrinkage of the muscle cells. As a result the percentage of water per kilogram of muscle cells (H_2O)_c is now 70.5 per cent, instead of the normal value of 73.3 per cent (Table IV).

In spite of the large volumes of extracellular fluid present in the skin and skeletal muscle, neither the cerebellum nor the cerebral hemisphere had increases in total water content. There were, however, small changes in the chloride and sodium content, which indicated small increase in the extracellular phase at the expense of the brain cells. This finding demonstrates the constancy with which the fluid of the brain is distributed even when the interstitial spaces of all other tissues of the body are filled with extracellular edema.

SUMMARY

Data on the water, protein, and electrolyte distribution in blood and tissues of an impounded dog with cholangitis, early biliary cirrhosis, and generalized anasarca are presented.

By analyses the total serum protein concentration of 4.22 Gm. per cent, of which 2.72 Gm. per cent was albumin and 1.5 Gm. per cent was globulin, could have been an important causal factor in the edema of the dog, but other factors must have been present.

With the presence of this type of edema the skin and muscle had increases in water content as much as 22.2 and 5.3 per cent, respectively. The large increases in sodium and chloride concentrations in the skin indicate that the increased water content was extracellular. The total bulk of a kilogram of muscle before the onset of disease was calculated to have increased during the disease 224 Gm. or to a total volume of 1,224 Gm., of which a 304 Gm. increase were attributed to the extracellular phase and a loss of 80 Gm. from the muscle cells. This is an example of edema and dehydration appearing simultaneously. In spite of the extracellular edema existing in skin and muscle, neither the cerebellum nor cerebral hemispheres showed any increase in total water content. This finding demonstrates the constancy with which the fluid of the brain is distributed even when the interstitial spaces of all other tissues of the body are filled with extracellular edema.

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SENSITIZATION OF Rh-POSITIVE PATIENTS BY THE Rh FACTOR

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THE discovery of the Rh factor and the recognition of the role that it plays in connection with transfusion have created certain practical problems. The fact is now recognized that when repeated transfusions of Rh-positive blood are given to an Rh-negative patient antibody response to the Rh antigen develops in approximately one in twenty-five patients. Instances in which antibodies develop after one transfusion also have been reported.¹ These antibodies may be bivalent (anti-Rh agglutinins) or univalent (blocking antibodies).² Further transfusions of Rh-positive blood into patients so sensitized result in hemolytic reactions. Approximately 90 per cent of all hemolytic post-transfusion reactions are caused by the Rh factor. It is also established that exposure of an Rh-negative woman of any age, from birth to the climacteric, to Rh-positive blood by transfusion may result in her inability to give birth to a normal infant. Furthermore, repeated pregnancies in an Rh-negative woman bearing Rh-positive children may sensitize the patient, even though clinically all of her children may have been apparently normal. In such a patient transfusion may be followed by a hemolytic reaction.

It is apparent that it becomes incumbent upon everyone ordering transfusions to understand the mechanism involved and to avoid the development of posttransfusion hemolytic reactions. All who select donors and perform tests necessary for the selection of a donor must revise their technique in the light of recent advances. A previous paper³ referred to various routines now followed. It was stated that the ideal procedure is to perform an Rh typing on every patient, as well as on every donor, and to use for transfusion only a donor belonging to the same Rh type as the patient, as well as one of the same ABO group. It was pointed out that perhaps in the future, and it is hoped in the not distant future, this will be the accepted technique. For the present it was advised that the blood of every patient be tested for the Rh₀ factor, that all those who react positively to this factor (85 per cent) be classified as Rh-positive patients, and that all those who react negatively be classified as Rh-negative patients. It was recommended also that the bloods of all donors be tested with Rh₀' serum and all those who react positively (87 per cent) be classified as Rh-positive donors, while those who react negatively be classified as Rh-negative donors. For transfusion of all patients classified as Rh positive Rh-negative donors should be used; for all Rh-negative patients the blood of Rh-negative donors should be selected. This routine will eliminate from the roster of Rh-negative donors all individuals who are positive either to the Rh₀ or the Rh' factor. This routine, however, deliberately disregards donors belonging

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to type Rh'', and they would be included in the roster as Rh negative. Such donors are rare, constituting only 0.6 per cent of the total, while Rh-negative patients constitute approximately 15 per cent of the total patients. Such a combination, namely an Rh'' donor (classified as Rh negative) and used for the transfusion of an Rh-negative patient with subsequent development of anti-Rh'' agglutinins, is very unlikely. On the other hand this idea fails to take into consideration the fact that when blood of Rh-positive donors is transfused into Rh-positive patients 50 per cent of all patients are Rh positive and belong to Type Rh₁. They lack the Rh'' factor just as do Rh-negative patients. The blood of approximately 28 per cent of all donors belongs to types Rh₂ and Rh₁Rh₂ and contains the Rh'' factor. Exactly how frequently sensitization results from such a combination remains to be seen; it probably is not common. The possibility exists, however, that it occurs more often than we now suspect and that the cases go unrecognized. This opinion is based on the fact that at present if Rh determinations are done on patients and donors prior to transfusion the test is merely for the Rh₀ factor. If both patient and donor are classified as Rh positive, and a posttransfusion reaction occurs, the reaction is considered pyrogenic in nature; thus, the diagnosis of a hemolytic reaction is dismissed, especially if jaundice or hemoglobinuria do not develop. Yet these clinical symptoms are not always present, and the diagnosis can be made only serologically. The following case illustrates the fact that Rh-positive blood transfused into an Rh-positive patient can cause sensitization, and a hemolytic posttransfusion reaction can result. This is the first completely proved case of Rh'' sensitization induced by transfusion in an Rh-positive patient; demonstrable antibodies were found in the patient's serum.

CASE REPORT

C. M., a white man, 25 years of age, was referred to me by Colonel E. S. Olson and Captain F. E. Fonseca, of the Medical Corps. While on army duty in India in 1942 the patient fell from a troop train and sustained compression fractures of the ninth dorsal and first lumbar vertebrae, injuring the spinal cord. He developed a spastic paraplegia. Because of involuntary micturition and defecation a suprapubic cystostomy and a perineal urethrotomy were done. In 1943, a laminectomy and exploration at the site of the spinal injury was performed but was not followed by any significant improvement. For the past two years he has been suffering from numerous decubitus ulcers which necessitated vigorous supportive therapy, especially in the form of repeated blood transfusions. The patient's blood was classified as Group AB, Rh positive. The first transfusion was given Oct. 15, 1944, and from then to March 28, 1946, or during a period of approximately one year and four months, he received a total of twenty-three blood transfusions and one plasma transfusion. On one occasion the transfusion was of 1,000 c.c., but on each other occasion 500 c.c. were given. For the first twenty transfusions Rh-positive blood was transfused.

In Table I is given the patient's transfusion history. Because of the reactions that followed the transfusions, and because these reactions were becoming more severe, I was asked to see the patient. Examination of his blood showed it to be Group AB, Subgroup A₂, type M, Rh₁, Hr positive. Examination of the patient's serum (Table II) showed the presence of anti-Rh'' agglutinins in a titer of 1:4. Anti-Rh antibodies of other types were not and,

TABLE I. TRANSFUSION HISTORY OF PATIENT (GROUP AB INTERNATIONAL)

DATE OF TRANSFUSION	Rh DETERMINATION OR TYPE	AMOUNT TRANSFUSED	POSTTRANSFUSION REACTION
11/15/44	Positive	500 c.c. blood	Urticaria
8/ 1/45	Positive	500 c.c. blood	No reaction
9/ 4/45	Positive	500 c.c. blood	No reaction
9/13/45	Positive	500 c.c. blood	Urticaria
9/19/45	Positive	500 c.c. blood	No reaction
10/ 3/45	Rh ₂	500 c.c. blood	Chill, cyanosis, urticaria
10/18/45	Rh ₁	500 c.c. blood	No reaction
10/ 3/45	Positive	500 c.c. blood	No reaction
11/21/45	Positive	500 c.c. blood	Temperature 103° F., mild urticaria
11/29/45	Positive	500 c.c. blood	Chill, temperature 102° F.
1/ 3/46	Positive	500 c.c. blood	No reaction
1/19/46	Positive	500 c.c. blood	No reaction
2/ 6/46	Rh ₁	500 c.c. blood	No reaction
2/ 8/46	Positive	500 c.c. blood	No reaction
2/11/46	Positive	500 c.c. blood	No reaction
2/13/46	Positive	500 c.c. blood	Chill, temperature 103° F.
3/11/46	Positive	500 c.c. blood	Temperature 102° F.
3/13/46	Positive	500 c.c. blood	Chill, temperature 104° F., urticaria
3/15/46	Positive	500 c.c. blood	Chill, temperature 105° F., urticaria
3/18/46	Positive	500 c.c. blood	Chill, temperature 104° F., urticaria
3/19/46	Positive	500 c.c. plasma	Urticaria
3/21/46	Negative	1,000 c.c. blood	Urticaria
3/22/46	Negative	500 c.c. blood	No reaction
3/28/46	Negative	500 c.c. blood	No reaction

TABLE II. TITRATION OF ABNORMAL ANTIBODIES IN SERUM OF PATIENT REPORTED

DATE OF TEST	TEST CELLS	DILUTION OF PATIENT'S SERUM				
		UNDILUTED	1:2	1:4	1:8	1:16
3/19/46 Tested by agglutination method	rh*	---	---	---	---	---
	Rh ₀	---	---	---	---	---
	Rh ₁	---	---	---	---	---
	Rh''	+	+	+	---	---
	Rh ₂	++	+	+	---	---
3/19/46 Tested by conglutination method	rh	---	---	---	---	---
	Rh ₀	---	---	---	---	---
	Rh ₁	---	---	---	---	---
	Rh''	+ ~ ±	± ±	±	tr	---
	Rh ₂	++	+	tr	---	---
3/27/46 Tested by agglutination method	rh	---	---	---	---	---
	Rh ₁	---	---	---	---	---
	Rh ₂	++	± ±	+	tr	---
3/27/46 Tested by conglutination method	rh	---	---	---	---	---
	Rh ₁	---	---	---	---	---
	Rh ₂	± ±	± ±	±	±	---

Tests were also made at other times, but the results are not given because they were essentially the same.

*Type rh is the short designation for Rh-negative blood.

of course, could not be present. No Rh₀ blocking antibodies were present. By the conglutination method performed in the test tube, tests also demonstrated the Rh'' specificity; the result was positive in a titer of 1:4. When tested

on the slide the reaction also was positive only against blood containing the Rh'' factor. It will be noted from Table I that the blood used for the first twenty transfusions was in each instance Rh positive. Ten of these were followed by no reaction whatsoever. Two were followed by urticaria, one by fever and urticaria, two by chill and fever, and four by chill, fever, and urticaria. The urticarial reaction was undoubtedly due to protein sensitization as evidenced by the fact that urticaria also developed after the administration of 500 c.c. of plasma. The six transfusions which were followed by a chill were undoubtedly hemolytic in nature. It is possible here to report the result of the examination of the blood of only three of the donors used. One belonged to type Rh₂ and transfusion of his blood gave the most severe reaction; two donors belonged to type Rh₁, and the transfusions of their bloods were followed by no reaction whatsoever. These results coincided with the fact that the patient's blood belonged to type Rh₁, and his serum contained Rh'' agglutinins. One or more of the first five donors had blood containing the Rh'' factor. This was the only factor lacking in the patient's blood; he became sensitized and developed anti-Rh'' agglutinins. The blood of the sixth donor belonged to type Rh₂, and its administration was followed by a severe hemolytic reaction. From then on every time blood containing the Rh'' was given a hemolytic reaction resulted. Every time blood, even though Rh positive, was of a type lacking the Rh'' factor no reaction followed.

This particular case is complicated by the fact that there was a protein sensitization as evidenced by the appearance of urticaria on five occasions. On one occasion this symptom followed the administration of plasma, which, of course, contained no red blood cells. After the blood was examined, and the fact that it contained anti-Rh'' agglutinins was discovered, only Rh-negative blood was used. Three such transfusions were given without hemolytic reaction. One of these transfusions consisted of 1,000 c.c. (500 c.c. of washed Rh-negative Group O cells and 500 c.c. Rh-negative Group AB whole blood). This was followed by the appearance of a few urticarial lesions but no other untoward symptoms.

It should be pointed out, however, that it is unnecessary to use Rh-negative blood for a patient such as this. In fact such procedure is uneconomical and depletes the bank of Rh-negative blood. In order to have Rh-negative blood available when it is actually needed, it should be used only for Rh-negative patients. Inasmuch as this patient belonged to type Rh₁, a donor belonging to type Rh₁ should be used. The supply of Rh₁ blood is plentiful because such donors constitute 50 per cent of the total, whereas Rh-negative donors constitute only 15 per cent.

This case illustrates that sensitization by the Rh'' factor can occur and does occur in Rh-positive patients belonging to type Rh₁, and that simply using Rh-positive blood donors for Rh-positive patients is not complete protection. To avoid this, one must carry out either one of two routines. The ideal procedure is to perform a complete Rh typing on every patient and only use blood of the same Rh type, or at least not to administer blood which contains an Rh factor lacking in the patient. An alternate and simpler routine

is to use the appearance of a posttransfusion reaction as a signal for careful investigation of the patient's blood. The first reaction is never, or hardly ever, fatal. Subsequent ones may be, although in this case the patient survived six hemolytic reactions. These reactions were most distressing and were becoming progressively more severe and more serious. If after the transfusion given Oct. 3, 1945, when the patient developed a chill and cyanosis, examinations had been performed, it would have become evident that the patient belonged to type Rh₁ and the donor to Rh₂, and the Rh'' agglutinins would have been detected. The next donor was Rh₁, and in all likelihood the one used following this was Rh₁ because no reaction occurred. The next two were followed by reactions, undoubtedly because the blood contained the Rh'' factor. It is persistence in giving transfusions in the presence of a reaction that leads to trouble and in some instances to the death of the patient. Fortunately this did not occur in this instance. For the average institution which lacks the necessary sera and the personnel to perform these tests, this alternative and simpler rule is recommended: namely, *as soon as a posttransfusion reaction occurs, give no further transfusion until a complete investigation has been made.* Such blood, if it cannot be examined at the institution where the patient is hospitalized, should be sent to those laboratories which specialize in this field. There is, however, a simple test which is helpful and should be used except in women prior to the climesterie.⁴ Prior to the transfusion a specimen of the patient's blood should be drawn in 3 per cent sodium citrate solution (one part citrate solution, nine parts blood). This specimen can be used for the customary typing and cross-matching tests. If a reaction occurs, a second similar specimen should be drawn during the reaction or shortly after it has subsided. These two specimens should then be centrifuged and the color of the supernatant plasma compared. If the color before and after transfusion is identical, the reaction was pyrogenic and not hemolytic. If on the other hand the color of the plasma of the posttransfusion specimen is deeper than that of the pretransfusion specimen, the reaction is hemolytic. No further blood should be transfused until the problem is clarified and the proper blood obtained.

SUMMARY

The Rh factor is responsible for approximately 90 per cent of all hemolytic posttransfusion reactions. If only Rh-negative blood is administered to patients who are Rh negative, sensitization by the Rh factor will be avoided in such patients. In certain cases when blood is administered to Rh-positive patients, sensitization may follow providing the patient lacks any of the three Rh factors and the donor's blood contains the factor or factors absent in the blood of the patient. A case illustrating this point is reported. The patient belonged to type Rh₁ and therefore lacked the Rh'' factor. Donor's blood containing the Rh'' factor had been administered and anti-Rh'' agglutinins developed in the blood of the patient. The donor whose blood produced the first hemolytic reaction experienced in this case belonged to type Rh₂, whereas subsequent transfusions with blood belonging to type Rh₁, or Rh negative, were followed by no hemolytic reaction. In order to protect these patients complete Rh

typing must be performed, and only blood of the same Rh type administered or the appearance of a reaction must be used as an indication for complete serologic examination of the patient's blood. After the appearance of one reaction, no further transfusion should be given until serologic examination shows that the reaction was not hemolytic in nature. Clinically, patients do not benefit from transfusion followed by a hemolytic reaction. Persistence in giving transfusion in spite of a reaction may be dangerous.

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THE TRAUMATIC EFFECTS OF POSITIVE INTRATRACHEAL PRESSURES

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THE use of positive pressure inhalational therapy has by now received considerable support. It apparently was used first as long ago as 1878 by Oertel¹ who employed 100 inspirations of air compressed to $\frac{1}{50}$ of an atmosphere excess pressure in the treatment of severe asthma. In 1897, Norton² reported the successful employment of positive pressure in the treatment of pulmonary edema caused by carbolic acid. Since then, Emerson,³ Poulton and Oxon,⁴ Barach,⁵ and others have recommended the use of positive pressure breathing in the treatment of bronchial asthma, chronic bronchitis, emphysema, and pulmonary edema caused by left-sided cardiac failure. Christie⁶ suggested that the maintenance of a positive intra-alveolar pressure throughout the respiratory cycle might diminish the tendency to pulmonary edema in patients poisoned with phosgene, and Barach⁵ indicated the importance of this procedure in the prevention and treatment of pulmonary edema caused by irritant war gases, referring to a series of patients with varying degrees of pulmonary edema due to inhalation of chlorine and nitric fumes which had been so treated with considerable success.

The employment of positive intratracheal pressure is not without potential hazard to the pulmonary tissues. Lilienthal⁷ noted interstitial emphysema in a patient receiving general anesthesia by insufflation when the intratracheal pressure was raised to 60 mm. of mercury. Ewald and Kobert⁸ found that air under a pressure of 40 mm. of mercury caused rupture of alveoli and pneumoperitoneum in rabbits. Joannides⁹ was able to force air or fixing fluid through pulmonary alveoli into the pulmonary circulation without producing apparent gross or microscopic injury to the alveolar walls, but he and Tsoulos¹⁰ found that when intrapulmonic pressures of from 60 to 100 mm. of mercury were attained in either normal or pneumonic dogs, interstitial pulmonary emphysema, pneumothorax, pneumoperitoneum, and air embolism invariably resulted. By means of a Van Allen carotid cannula, Polak and Adams¹¹ also demonstrated the occurrence of air emboli in animals subjected to increased intrapulmonic pressures.

At somewhat lower levels of pressure, Marcotte and associates,¹² working with dogs, found that intrabronchial pressures of 24 mm. of mercury maintained for periods of from 16 to 110 minutes were routinely accompanied by interstitial emphysema, usually along the great vessels at the hilum of the lungs but, on two occasions, spreading along the trachea into the neck; in one

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animal interstitial emphysema resulted at a pressure of only 18 mm. of mercury. They found that such emphysema occurred in cats at from 16 to 20 mm. of mercury. Similarly, Rasmussen and Adams,¹² in single acute experiments and in chronic intermittent experiments in dogs over periods of from one week to eleven months, found varying degrees of gross and microscopic emphysema and, in a few instances, air embolism and pneumothorax at pressures of from 30 to 35 mm. of mercury at the carina.

The occurrence of such effects of increased intrapulmonic pressure in normal animals raises the question whether traumatic tissue damage of even greater degree or at even lower levels of pressure might not result in lungs already the site of damage, as indicated by the presence of pulmonary edema. A comparison was therefore made between the effects of increased intratracheal pressures in normal dogs and in dogs with pulmonary edema produced by the inhalation of phosgene. Particular note was made of the pressures required to produce arterial air emboli in the two groups of dogs.

EXPERIMENTAL

One group of dogs was gassed with phosgene to permit the development of pulmonary edema prior to the remainder of the experimental procedure. After sufficient time elapsed, these and the normal control dogs were anesthetized by the intraperitoneal injection of 20 mg. of nembutal per kilogram of body weight. Each dog was then tracheotomized, and a Y tube cannula was inserted into the trachea. One arm of the cannula was attached to a source of air pressure and the other to a mercury manometer. Arterial and venous blood pressures were recorded directly from cannulae inserted into the femoral artery and vein. After intravenous administration of 100 mg. of heparin, a Van Allen cannula was inserted into the right carotid artery to permit visualization of air emboli flowing through the artery. A tambour recording pneumograph was placed around the chest. The head of some of the animals was elevated to facilitate trapping of the air emboli in the carotid cannula.

The intratracheal pressure was then increased by 10 mm. of mercury; this was maintained for a period of ten seconds. Several minutes were then allowed for return of respiration and blood pressure to normal. In this manner, the lungs were subjected to successive increments of positive intratracheal pressure for periods of ten seconds each, with intervening rest periods of several minutes. In some instances, the experiment was concluded at 30 or 50 mm. of mercury and the animal sacrificed for gross and microscopic study. In the majority, the experiments were concluded when air emboli were noted in the carotid trap, when gross interstitial emphysema was observed through the cervical incision, or upon death of the animal.

RESULTS

Normal Dogs.—With elevation of the intratracheal pressure, there occurred a drop in arterial blood pressure, an elevation of venous pressure, and cessation of respiration (Fig. 1). These effects persisted while the increase in intra-

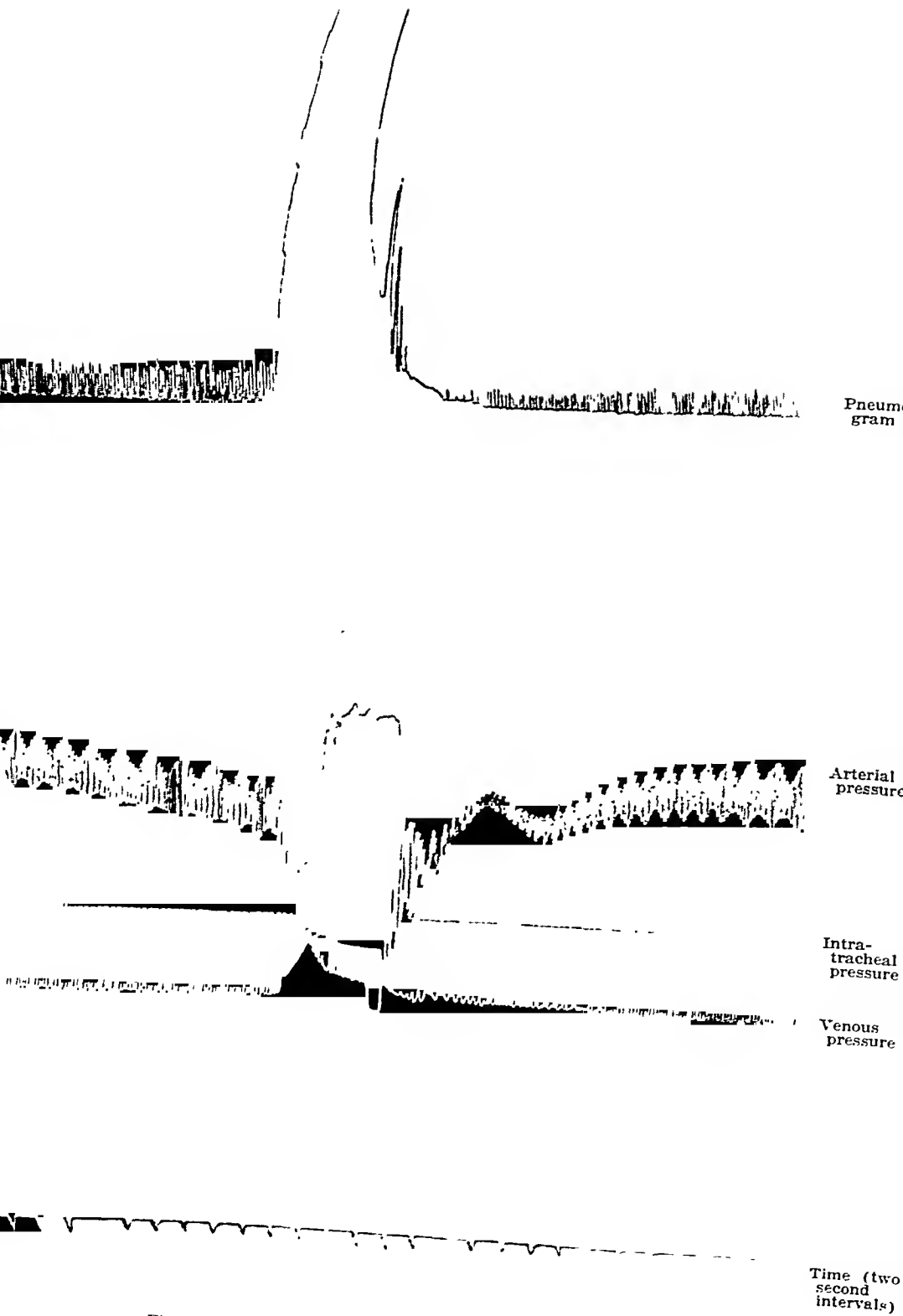


Fig. 1.—Normal dog, 60 mm. Hg intratracheal pressure.

tracheal pressure was maintained. When the increased pressure was discontinued and the intratracheal pressure allowed to drop to normal, the venous blood pressure immediately returned to normal; this was followed within a few seconds by resumption of normal respirations and a gradual return of the arterial pressure to normal level at about fifteen seconds.



Fig. 2.—Interstitial emphysema in dog subjected to elevation of intratracheal pressure of 90 mm. of mercury. Note compression of pulmonary artery. The longitudinally cut bronchus is at the right margin. Hematoxylin and eosin ($\times 40$).

In three dogs (heads elevated), air emboli appeared in the carotid trap at increased intratracheal pressures of 80, 90, and 90 mm. of mercury, respectively. In a fourth dog (head not elevated), gross mediastinal emphysema was observed through the cervical incision at 80 mm., although no air emboli were noted in the carotid trap; in addition, autopsy revealed pneumothorax and pneumoperitoneum. Microscopic examination of the lungs of these four dogs revealed moderate dilatation of the terminal respiratory passages, often associated with focal vesicular emphysema. Only very slight, focal tearing of

alveolar septa or intra-alveolar hemorrhage was noted; in general, these were absent. The most notable feature was the presence of numerous, interstitially located bubbles of air; these were especially prominent in the larger septa toward the hilus of the lung, often associated with resulting compression of the adjacent pulmonary artery (Fig. 2). Occasionally there was associated interstitial hemorrhage.

Two dogs were sacrificed after the intratracheal pressure had been elevated to 50 mm. of mercury. No carotid air emboli had been noted in these animals, nor was there gross mediastinal or cervical interstitial emphysema. Microscopically, however, the lungs showed changes similar to, although somewhat less severe than, those in the preceding group of dogs; in particular, foci of interstitial emphysema were fairly numerous within the interlobular septa adjacent to the pulmonary arteries. One dog was sacrificed after being subjected to an increased pressure of 30 mm. of mercury; no gross or microscopic changes were observed.

Dogs With Pulmonary Edema.—In twelve dogs in which a substantial degree of pulmonary edema had developed as a result of the gassing with phosgene, the effect of increased intratracheal pressure on respiration and on the arterial and venous blood pressure was indistinguishable in type or degree from that observed in the normal dogs. In three animals of this group (heads elevated), carotid air emboli were observed at increased intratracheal pressures of 80, 110, and 110 mm. of mercury, respectively. In another five dogs (heads not elevated), one exhibited carotid air emboli at a pressure of 90 mm. of mercury, two developed gross interstitial emphysema at a pressure of 100 mm. of mercury, and the remaining two exhibited neither gross interstitial emphysema or carotid air emboli but failed to resume normal respiration upon release to normal of the intratracheal pressure which had been raised to 100 and 110 mm. of mercury, respectively. In these eight dogs, in addition to the substantial degree of pulmonary edema and other changes typical of phosgene poisoning, there were changes in the lungs attributable to the increased intrapulmonic pressure which were essentially identical with those observed in the control dogs subjected to similar levels of increased intrapulmonic pressure. It appears likely that the cause of death in the last two dogs was air embolism to the brain despite the failure of the carotid trap to visualize any air emboli.

In two dogs with pulmonary edema, increase in intratracheal pressure to 50 mm. of mercury resulted in somewhat less severe effects than in the controls at this pressure, both showing slight focal dilatation of terminal air passages and relatively few foci of interstitial emphysema within the lungs on microscopic examination. Two dogs of the group with pulmonary edema subjected to a pressure of 30 mm. of mercury showed no gross or microscopic changes in the lungs other than those caused by the phosgene.

DISCUSSION

The results observed indicate that the physiologic response of arterial and venous blood pressure and respiration to increased intratracheal pressure is essentially the same in dogs with pulmonary edema resulting from gassing

with phosgene as it is in normal dogs. Similarly, air embolism and/or gross mediastinal and cervical interstitial emphysema occur at about the same level of increased intratracheal pressure in the two groups of dogs. Both the normal dogs and those with pulmonary edema also exhibit the same degree of microscopic pathologic changes as a result of the increased pressure. The lack of significant apparent damage to the intra-alveolar septa as a result of the increased pressure is of interest; this is in agreement with the observations of Hartroft¹⁴ in human emphysema and in substantial agreement with the findings of Rasmussen and Adams¹⁵ in their experimental animals. Macklin¹⁶ has inferred that, as a result of the alveolar distention, numerous fine ruptures occur in the base of alveoli abutting against the vascular sheaths rather than in the lateral alveolar walls, because of the absence of pores at the former site, and by the breaking through of air from the alveoli into the interstitial tissue in this manner explains the pathogenesis of the interstitial emphysema.

In the present study, damage was observed to occur to the lungs, both in the normal dogs and in those with pulmonary edema, at a pressure of 50 mm. of mercury but not at 30 mm. However, in view of the shortness of the bursts and in view of the findings of other investigators previously quoted, it would appear that the therapeutic use of positive pressure inhalational therapy should not exceed pressures of from 15 to 20 mm. of mercury. It would also appear that there is no greater hazard to pathologic than to normal pulmonary tissue from this procedure.

SUMMARY

1. Increased intratracheal pressure in dogs, under the conditions of this study, resulted in drop in arterial blood pressure, elevation of venous pressure, and apnea. There was no essential difference noted between the response of normal dogs and those with pulmonary edema resulting from inhalation of phosgene.

2. Air embolism and/or gross mediastinal and cervical interstitial emphysema occurred at about the same level of intratracheal pressure in the two groups of dogs.

3. The microscopic pathologic changes, other than the lesions caused by inhalation of phosgene in the one group of dogs, were also of essentially the same magnitude in the two groups of animals. These changes consisted of moderate dilatation of terminal respiratory passages, focal vesicular emphysema, and interstitial emphysema, first noted microscopically at a pressure of 50 mm. of mercury in this study. Gross mediastinal and cervical interstitial emphysema and carotid air emboli became apparent at pressures of about 80 to 110 mm. of mercury in both the control animals and in those with pulmonary edema.

4. The findings indicate that there is no greater hazard as a result of positive pressure inhalational therapy to lungs with edema than to normal lungs.

Acknowledgment is made to Mrs. Harriet Kriete, for assistance in gassing the dogs with phosgene.

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LABORATORY METHODS

AN APPARATUS FOR MEASURING THE RETAINED DOSE OF INHALED SUBSTANCES

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THE biologic effect of a given substance is usually assayed by determining that quantity per unit of body weight which produces specific changes under well-defined conditions. Such assay is not difficult for substances which may be injected or ingested without changes in physiologic activity, since the administered dose can be accurately measured. However, for inhaled substances the determination of dose is much more difficult.

To circumvent technical difficulties in actual dose measurement, inhaled substances have been assayed by exposing animals for a measured period of time to a known concentration of the test material. Since the work of Haher,¹ such exposures have been measured in terms of the lethal index or Ct (the product of the concentration of material times the minutes of exposure) in an effort to obtain some measure of dose. However, the concentration-time product is merely a measure of physical exposure and can never constitute an accurate estimate of the dose retained. The latter depends not only on the Ct but also on the percentage retention and the respiratory intake during exposure and is given by

$$D = \frac{VTC\alpha}{W}$$

where D = the retained dose in milligrams per kilogram, V = respiratory intake during exposure in liters per minute, T = exposure duration in minutes, C = concentration of material inhaled in milligrams per liter, α = percentage of inhaled material retained, and W = body weight in kilograms. Since both the percentage retention and the respiratory minute volume during exposure may vary widely when animals of the same or different species are exposed, animals exposed simultaneously to the same Ct may retain markedly different doses. Consequently, it is not surprising that extensive individual and species variations in response to different inhaled toxic materials have been observed when the Ct is the index of dose administered.

For the proper evaluation of the effect of inhaled substances the actual dose retained must be measured. Were this possible, several lines of investigation would be facilitated. For example, in the evaluation of aerosol therapy² the efficacy of a given inhaled dose of a therapeutic agent could be compared with

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that of a dose administered by one of the conventional routes. In addition, volatile industrial hazards could be assessed more accurately. Finally, in studies of the pulmonary blood-air barrier it would be possible to compare the amount retained in the lung with that transported across the semipermeable membrane.

Tobias, Patt, Swift, and Gerard^{2*} developed a method for measuring the retained dose of an inhaled substance, which involved the use of the following: a dynamic chamber from which the animal withdraws air containing the test material at known concentrations, a valve system to direct the flow of air, an absorber to remove quantitatively any of the material which is expired, and a spirometer to record the volume of the expired air. The product of the chamber concentration times the volume inspired gives the quantity of the test substance removed from the chamber. The difference between this and the quantity in the absorber, as determined by chemical analysis, is the dose retained by the animal. Critical evaluation of their early experiments reveal that, although the method was correct in principle, there were several sources of error in the apparatus employed. Moreover, it was not applicable to unanesthetized animals without tracheal intubation.

As a result an apparatus has been developed which has the following characteristics:

1. All surfaces which come into contact with the test substance are made of glass or other chemically inert materials.
2. The resistance at ordinary respiratory air flows is low.
3. Condensation of expired moisture on surfaces of the valves is prevented, thus minimizing adsorption and surface hydrolysis.
4. Unanesthetized (or anesthetized) animals can be used with minimal discomfort.

DESCRIPTION OF APPARATUS

Specific dimensions given herein are not critical. The size of the apparatus should depend upon the species studied. The measurements given have been found satisfactory for the dog and the goat.

Valves.—Since the conventional valves contain some rubber, metal, or plastic materials which react with many chemical substances, valves consisting entirely of glass were developed. After preliminary trials with several designs, the present type, using standard tapered joints as valve housings, was selected because it combines simplicity of construction with low resistance to ordinary air flows (Fig. 1).

A circular glass disc (*A* in Fig. 1), 24 mm. in diameter and 1.5 mm. thick, is ground on one surface until an airtight seal is obtained, when the disc is seated on a similarly ground end of the male half of a 24/12 standard tapered, glass joint (*B* in Fig. 1). The grinding is accomplished in a few minutes by rubbing the surface of the disc and the valve seat on a piece of plate glass, using carborundum in water as the grinding agent. The cross sectional diameter of

*The present authors wish to acknowledge their indebtedness to the early collaboration of Dr. R. W. Gerard, Dr. H. M. Patt, and Miss M. N. Swift. Due to the exigencies of war-time research, these investigators were unable to participate in the development of the new apparatus, but their earlier assistance, planning as well as technical, was invaluable.

the airway should be large enough to pass the expected airflows with minimal resistance, but the total volume of the system should be as small as possible to diminish rebreathing by decreasing the dead air space.

As can be seen from Fig. 1, there is a circumferential dilation of the glass tubing for a short distance distal to the female half of the joint. Thus, the valve disc, when in position in the closed joint, can move in this dilated area during each respiratory cycle. When the disc is seated on the ground end of the male half of the joint, the valve is closed; when it is moved away from the end of the male half, the valve is open. The discs can shift sufficiently in this dilated space to allow passage of air with very little resistance (see paragraph on Resistance of Combined Valves and Absorber). The valves function most efficiently and rapidly with the long axes vertical and valve seats below, so that the discs are seated by gravity. Thus, the resistance which the valves offer is determined primarily by the weight of the discs.

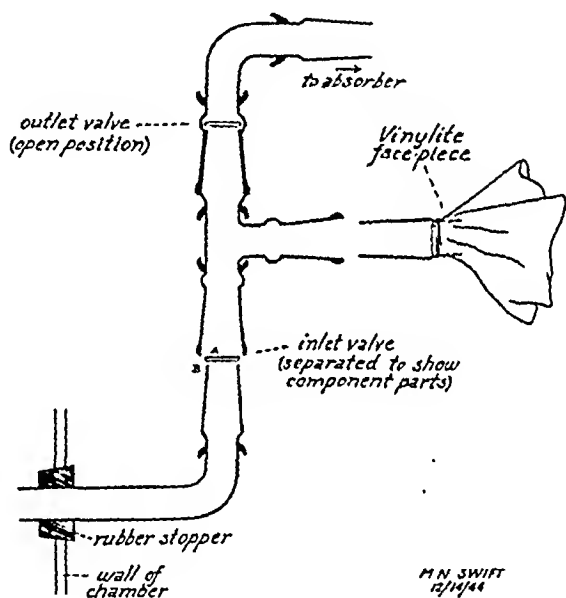


Fig. 1.—Valves and facepiece.

The entire valve assembly is wrapped with appropriate lengths of No. 24 gauge Nichrome wire insulated with asbestos tape. By regulating the current through the wire with a rheostat, the temperature of the glass is maintained slightly above body temperature to prevent condensation of water vapor from the expired air. Thus adsorption and hydrolysis on the glass surfaces are minimized. Sticking of the valves because of accumulated moisture is also avoided.

Absorber.—The absorber (Fig. 2) consists of a vertical, 48 mm. outer diameter glass cylinder, tapered to a 3 mm. stopcock below and to a ground glass stopper above. The inlet tube (20 mm. outer diameter) is connected to the expiratory valve by a ground glass joint and enters the absorber about 5 cm. above the stopcock. Then it bends sharply downward to terminate in a rounded blind end

which is perforated with a sufficient number of holes (about fifteen), 3 mm. in diameter, to permit free flow of expired gas into the absorber. The absorber is filled to a height of 23 cm. with 4 mm. solid glass beads which, when wet with a suitable absorbent solution, provide a large absorbing surface. However, since beads would plug the inlet holes, the lower 9 or 10 cm. of the absorber are filled with readily draining solid glass spirals. These are of such size and shape that they cannot obstruct the inlet openings. The outlet tube (20 mm. outer diameter) leaves the absorber above the level of the glass beads and leads to a recording spirometer.

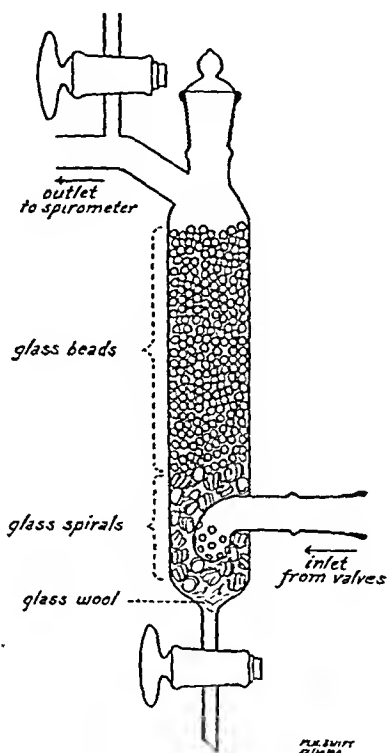


Fig. 2.—Low resistance absorber.

Immediately before use the inlet tube is closed with a glass stopper and the absorber filled with the best solvent for the substance being studied. Then it is shaken vigorously to remove any air bubbles trapped among the beads which might prevent some of the surfaces from becoming covered with solvent, and the solvent is drained from below. The stopper is removed and the inlet joint dried and attached to the valve outlet. After use the absorber is disconnected from the valves and the glass stopper replaced in the inlet. The beads, spirals, and inlet tube are washed thoroughly by repeated rinsings introduced from above. The several washings are drained through the bottom stopcock into a volumetric flask, and an appropriate aliquot is analyzed. The absorber need not be cleaned additionally for the next sampling. All that is required is to dry the inlet tube and to repeat the steps outlined.

Facepiece (Figs. 1 and 3).—The unanesthetized animal may be attached to the apparatus by means of a roughly cone-shaped, Vinylite facepiece which is sealed to the male half of a ground glass joint. The animal's mouth is held closed with Ace elastic bandage, care being taken not to occlude or to compress the nostrils, and the facepiece is slipped over the taped snout until the inner end of the glass joint nearly touches the animal's nose. More elastic bandage is wrapped around the facepiece and snout, thus diminishing the dead space. By means of the joint the animal is attached to the valve system (Fig. 3). Thus, the mouth is held closed to prevent leakage of saliva into the system, and an air-tight connection between the animal and the apparatus is achieved. The procedure apparently causes little discomfort. Some untrained animals require no restraint and stand quietly breathing into the apparatus.

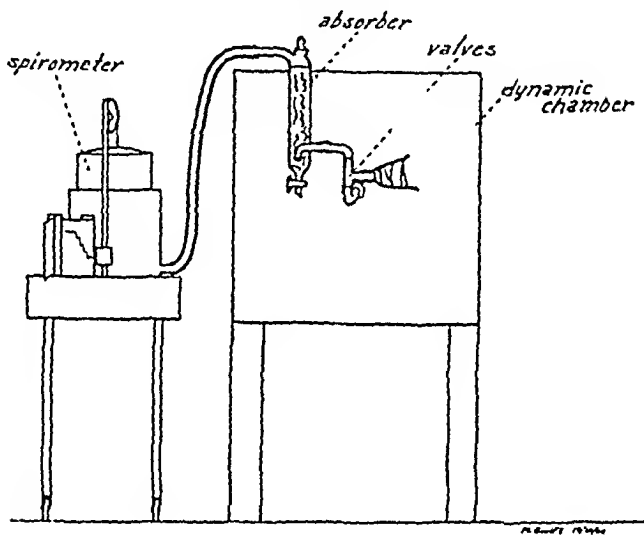


Fig. 3.—Assembled apparatus.

With care the facepiece can be applied on dogs and goats without leakage at the usual air flows. A modification of the facepiece which permits no leakage has been developed for use in monkeys.⁵ However, as yet, no chemically inert, leak-proof facepiece has been found for use on rabbits.

In experiments in which severe hyperpnea may occur, the increased pressure of expiration may cause significant leakage. Therefore, it has been found advisable to test for leakage before experiments.⁴ In this test the entire apparatus is assembled and the animal attached as usual. However, the inlet valve instead of being connected to the experimental atmosphere is joined to a calibrated spirometer. The animal then inspires air from one spirometer and expires into a second. Any leak would be indicated by a discrepancy between the volume changes in the two spirometers. If there is no leak, the inlet valve can be switched immediately to the experimental atmosphere and the experiment begun. When significant leakage has been noted, substitutes for the facepiece have been employed successfully with the valves and absorber. The upper

respiratory tract can be short-circuited by tracheal or nasal intubation,⁴ both under general anesthesia, or by tracheotomy under local anesthesia.⁵

TESTS OF APPARATUS

Valve Efficiency.—To test the valves for leakage, the facepiece was replaced by a calibrated 50 c.c. syringe joined to the system by a rubber stopper. By repeated strokes of the syringe plunger measured amounts of air were drawn through the inlet valve and expelled through the outlet valve into a spirometer previously calibrated with the same syringe. With no leakage there should be 100 per cent recovery of the air pumped through the valves. Repeated tests (Table I) revealed essentially zero leakage.

TABLE I. TESTS FOR VALVE LEAKAGE

NUMBER OF SYRINGE STROKES	TOTAL VOLUME PUMPED BY SYRINGE (C.C.)	VOLUME RECOVERED IN SPIROMETER (C.C.)	DIFFERENCE	
			C.C.	%
60	3000	3000	0	0
60	3000	2980	20	0.7
60	3000	3000	0	0
60	3000	3000	0	0
60	3000	3000	0	0
59	2950	2950	0	0
58	2900	2900	0	0
40	2000	2000	0	0
40	2000	1990	10	0.5
40	2000	2000	0	0
20	1000	1000	0	0
20	1000	1000	0	0
20	1000	1000	0	0

Absorber Efficiency.—Several types of experiments were performed to establish the efficiency of the absorber. The test substance was a gas containing chloride. Chloride ion in the absorber (and in the standard Vigreux bubblers used for comparison) was titrated potentiometrically in a modified Pineof cell.⁶

Three one-liter samples of air, containing from 7.3 to 10.8×10^{-5} mols of Cl per liter, were drawn, first through the absorber (beads wetted with 5 per cent NaOH) and then through a standard Vigreux bubbler in series with it. With flow rates of from 750 to 1,500 c.c. per minute no Cl was found in the second bubbler in six trials. Therefore, the bead absorber must have removed all the test gas from the air passing through it.

Again by steady suction, nearly simultaneous, three liter samples of Cl laden air were drawn from a dynamic chamber, one through the valves and bead absorber and another through a standard bubbler. In Table II recovery by the bead absorber and bubbler are compared. The percentage difference be-

TABLE II. CHLORIDE RECOVERY BY BEAD ABSORBER AND STANDARD BUBBLER (STEADY GAS FLOW)

CL RECOVERY		DIFFERENCE	
BEAD ABSORBER (MOLS $\times 10^{-5}$ PER LITER)	STANDARD BUBBLER (MOLS $\times 10^{-5}$ PER LITER)	MOLS $\times 10^{-5}$	%
17.75	17.70	0.05	-0.2
17.34	17.53	0.19	-1.0
17.70	17.44	0.26	+1.4

tween the two sets of values is small enough to have resulted from a summation of possible errors in titrations or volume measurements.

To approximate more closely the conditions of an actual experiment, the valves, absorber, and spirometer were set up in series. The chloride containing gas was pumped through the apparatus by a 50 c.c. syringe (simulating a breathing animal), from a chamber the gas concentration of which was simultaneously and independently analyzed. The data in Table III further establish the efficiency of the bead absorber. Chloride recovered from the absorber is seen to have agreed very well with the value predicted from standard chamber analysis.

TABLE III. CHAMBER CHLORIDE CONCENTRATION DETERMINED BY STANDARD BUBBLER AND BEAD ABSORBER WITH VALVES INTERPOSED (INTERMITTENT GAS FLOW)

VOLUME OF GAS PUMPED (C.C.)	CL CONCENTRATION IN CHAMBER (MOLS $\times 10^{-5}$ PER LITER)	HEAD ABSORBER CL (MOLS $\times 10^{-5}$ PER LITER)		DIFFERENCE	
		EXPECTED	MEASURED	MOLS $\times 10^{-5}$ PER LITER	%
3,000	5.67	17.01	16.98	0.03	-0.2
3,060	5.51	16.85	16.88	0.03	+0.2
3,050	5.51	16.80	16.83	0.03	+0.2
2,980	5.21	15.53	15.61	0.08	+0.5
2,980	5.13	15.29	15.35	0.06	+0.4
3,000	5.14	15.42	15.66	0.24	+1.5

Resistance of Combined Valves and Absorber.—To measure resistance of the apparatus at normal air flows, a calibrated membrane manometer was connected between the inspiratory valve and the facepiece. Thus, in addition to the spirometer measurement of tidal air and respiratory rate, a record could be obtained of pressure changes during respiration. After the temperature of the heating coil was adjusted and the beads in the absorber were moistened, dogs were permitted to breathe into the apparatus through the usual facepiece.

TABLE IV. RESISTANCE OF COMBINED VALVES AND ABSORBER

RESPIRATION PER MINUTE	TIDAL AIR (C.C.)		INSPIRATORY PRESSURE (MM. H ₂ O)		EXPIRATORY PRESSURE (MM. H ₂ O)	
	MIN.	MAX.	MIN.	MAX.	MIN.	MAX.
12	148	390	4.4	4.4	9.0	12.8
12	117	234	4.4	4.4	6.7	12.2
8	179	312	4.0	4.4	7.7	13.4
10	273	468	4.5	12.2	12.2	17.5
10	214	308	4.2	4.4	10.2	13.2

From the data in Table IV it is clear that both the inspiratory and expiratory pressures at different respiratory rates and tidal air flows are no greater than the normal intratracheal pressures. Therefore, the entire system, facepiece, valves, wet absorber, and spirometer, offers but slight resistance to normal respiration.

DISCUSSION

The apparatus described for determining retained doses of inhaled substances fulfills the essential specifications. All parts coming into contact with

respired mixtures are constructed of glass or other chemically inert materials. No condensation of exhaled water vapor can occur within its channels. Since the valve flaps are light in weight, since the diameter of the tubing is relatively large, and since the absorber utilizes a large wet surface rather than a liquid column, the resistance offered by the apparatus at normal respiratory flows is low. Unanesthetized animals can be used with a minimum of discomfort.

A factor to be considered is the quantitative relation between the volumes of inspired and expired air, because the volume breathed during exposure is measured as expired air. Actual measurements done in connection with leakage tests (see paragraph on Valve Efficiency) have detected no significant difference. It is, however, obvious that when an alkaline solution is used in the absorber CO_2 will be removed from the expired air. Consequently, since the animal consumes oxygen, the expired volume will be smaller than the inspired. The discrepancy would be about 4 to 6 per cent and will tend to make the retained dose too low. If greater accuracy is required, the discrepancy can be corrected approximately by calculation or accurately by determining the oxygen content of the expired air.

The valves, it should be recalled, constitute two outlets of a glass tube with a third outlet where the facepiece attaches. Thus, the volume delimited by the inspiratory valve disc below, the expiratory valve disc above, and the facepiece constitutes a "dead space" through which the animal must breathe. Consequently, at the end of each expiration the concentration of test substances in the dead space will be somewhat less than that in the chamber, the extent of the dilution depending on the concentration of test material in the expired air and the relationship between the volume of the dead space and the animal's tidal air. Conversely, during inspiration, as the experimental mixture is drawn from the chamber, the concentration in the dead space is increased by an increment which depends also on the dead space-tidal air relationship. At the end of inspiration some of this more concentrated mixture is expired into the absorber without coming into contact with the animal. As a result analysis of the absorber contents will yield a somewhat excessive estimate of the amount of material to which the animal was exposed, and if not corrected, the apparent percentage retention will be too low. On the other hand the concentration to which the animal is exposed will be less than that in the chamber due to the dilution in the dead space by the expired air. The detailed mathematic treatment and derivation of a correction factor for calculating the true percentage retention and the actual exposure concentration appear elsewhere.⁵ It is wise to reduce the dead space as much as possible by using short standard tapered joints. However, it should be emphasized that the existence of the dead space has no influence on the determination of the retained dose.

CONCLUSIONS

1. An apparatus has been constructed which permits accurate measurement of the actual doses of certain inhaled substances retained by unanesthetized animals.
2. The percentage of inhaled material retained may be calculated readily.

3. There is very little resistance to normal respiratory air flows.

4. The essential parts of the apparatus are entirely of glass and easily obtainable.

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THE RELATIVE EFFICIENCY OF WATER CENTRIFUGAL SEDIMENTATION AND OTHER METHODS OF STOOL EXAMINATION FOR DIAGNOSIS OF SCHISTOSOMIASIS JAPONICA

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THE demonstration of the eggs of *Schistosoma japonicum* in American personnel under clinical observation for suspected schistosomiasis frequently entails numerous stool examinations. Patients with light or subclinical infections or those following inadequate treatment may have repeatedly negative stools by the ordinary methods of examination. Initial treatment or retreatment is usually not given unless the stools are positive. It is, therefore, desirable to employ methods with the highest degree of efficiency in detecting eggs for diagnosis and subsequently in the evaluation of therapy. During the past year we have examined more than 25,000 stool specimens from 800 patients with suspected or proved schistosomiasis japonica. In the course of these examinations the relative efficiency of various stool techniques was studied. The purpose of this paper is to present a simple rapid method of stool examination which we have found to be more efficient in detecting eggs of *S. japonicum* than other routine or special methods currently employed.

MATERIAL AND METHODS

Each initial stool specimen from all patients suspected of having schistosomiasis was examined by direct smear, zinc sulfate centrifugal flotation, brine flotation, and the modified centrifugal water sedimentation method described herein. All subsequent stool specimens were examined by direct smear, special attention being given to select a portion containing blood or mucus if present, and by centrifugal water sedimentation. A selected number of positive specimens was submitted to comparative study by the acid-ether, zinc sulfate, direct smear, and centrifugal water sedimentation methods of stool examination as well as several modifications of these techniques.

Faust and Meleney,¹ in 1924, described a water sedimentation method of stool examination for the demonstration of eggs and miracidia of *S. japonicum*. This method requires the use of the whole fecal specimen and repeated washing and gravity sedimentation in glass containers of from 1,000 to 2,000 c.c. capacity. The major value of this method apparently lies in the large amount of feces employed, but it is not practical for routine use in large numbers of examinations because of the size of the vessels required and the time consumed in its use (from one to two hours or more before final sediment is ready for examination). However, it may be of value in an individual case with extremely few eggs if stools are repeatedly negative by other methods in which relatively small amounts of feces are used.

Modified Water Centrifugal Sedimentation Method.—

1. Place from 10 to 15 Gm. of the feces in a 125 c.c. Erlenmeyer flask containing about 100 c.c. of lukewarm tap water. Insert a No. 4 rubber stopper and shake vigorously for one or two minutes, or until the particles are in fine suspension.

2. Strain the emulsified feces through two layers of wet gauze into a 50 c.c. centrifuge tube with teated bottom (tube dimensions: height, $4\frac{3}{4}$ inches; diameter, 1 inch).

3. Centrifuge at 1,500 r.p.m. for thirty seconds (brake of centrifuge applied gradually).

4. The supernatant fluid is decanted and tap water of about 40° C. is added to the 50 c.c. mark. Insert a rubber stopper and shake several times until the sediment is finely dispersed. The warm water causes a scum to rise to the surface. This is desirable.

5. Repeat procedures 3 and 4 until the supernatant fluid is clear; three washings and three centrifugations are usually sufficient.

6. After the last washing and centrifugation the clear supernatant fluid is poured off and the packed sediment remains. If the finger is gently tapped against the side of the tube, the packed sediment is dispersed and settles to the bottom of the tube by gravity within a minute.

7. With a rubber-bulbed pipette place 4 drops of mixed sediment on a glass slide and cover with a large cover slip size 22 by 40 mm. Make at least two slides.

8. Examine under low-power objective of the microscope for eggs of schistosoma. *S. japonicum* eggs are oval, 70 to 100 by 50 to 60 μ , may or may not have a rudimentary spine, and in blue-white light appear greenish orange. *Schistosoma mansoni* eggs are elongated, 114 to 175 by 45 to 68 μ , and have large lateral spines. In both species, if the ova are viable, the miracidia will exhibit movements within the eggshells, and the activity of the flame cells may be seen under higher magnification.

9. If the slides are negative for schistosoma ova, add about 10 drops of water to the remaining sediment in the tube, leave overnight in a warm place, and examine the following day for miracidia. The entire contents of the tube can be placed on a large slide and examined under a dissecting microscope. This phase of the examination requires less than two minutes.

10. Excluding procedure 9, not more than five minutes are required for the examination of each slide, and not more than from fifteen to twenty minutes are required from procedures 1 through 8.

Summary of the Method:

1. A large sample of the feces is used; this is especially valuable in light infections.

2. Viability of the ova is determined.

3. Rapidity of method—report can be rendered within fifteen minutes.

4. A hatching test is a part of the method.

5. Other ova, cysts of protozoa, and larvae of helminths may also be found and identified. (For protozoan cysts add a drop of Lugol's or D'Antoni's iodine to slide.)

6. Preserved concentrated specimens can be made quickly by adding 10 per cent formalin to the sediment in the tube at the end of the fresh examination.

7. No chemical reagents are required; minimum standard laboratory equipment is used.

Comparative Results of Stool Examinations by Water Centrifugal Sedimentation and Other Methods.—

1. *Direct Smear:* 1,026 consecutive fecal specimens (171 patients) not containing any grossly visible blood or mucus were examined simultaneously by direct smear and centrifugal sedimentation. These patients all had received treatment previously and were under observation for the evaluation of success or failure of various forms of therapy. Patients who were found to have positive results were examined daily until another positive stool was found. Results in ten patients were found positive by direct examination of fecal material free of grossly visible blood or mucus. Results in these were all positive in water centrifugal flotation. In addition, results in thirty-nine patients were found positive by the latter method making a total of forty-nine positive cases detected by centrifugal water sedimentation or a relative efficiency of 4.9:1 for this method compared to direct smear examination. This particular run is considered a good test of both methods since all patients had been treated previously, and those with positive results were passing only a few eggs. In view of the fact that all patients with schistosomiasis in Army personnel diagnosed overseas or in this country will have received one or more courses of treatment, it may be expected that the majority of such patients still having active infections will escape detection by direct smear examination if no mucus or blood is present in the feces. The conditions in this test run will simulate those faced by physicians and laboratories in civil or veteran's facility practice.

2. *Examination of Mucus or Bloodstreaked Mucus by Direct Smear:* All stool specimens were examined carefully by inspection of the surface and crevices for the presence of areas of bloody or mucoid material. Such material was placed on several slides and examined microscopically. Of fifty consecutive specimens from different patients whose stools contained bloody mucus, forty-five (or 90 per cent) were positive for eggs of *S. japonicum* on direct smear examination of this material. Usually a large cluster of eggs was readily found under low-power magnification within a minute. Of the forty-five specimens which were positive on direct examination, forty-two were also positive by water centrifugal sedimentation performed on a portion of the same fecal specimen but from an area not containing visible mucus or blood. In addition, one of the five bloody mucus specimens which were negative by direct smear was positive by water centrifugal sedimentation. Actually, for diagnosis in this group of fifty patients, it would have been necessary to run by sedimentation only the five specimens which were negative on direct examination of bloody mucus. The total positive results in the group would

then be forty-six (or 92 per cent), of which direct smear alone had detected 97.8 per cent of the positive results. In this group of fifty 86 per cent of the total patients were detected by centrifugation alone.

Consideration of the findings discussed in the preceding two paragraphs have these practical implications. First, careful gross examination of stool specimens for the presence of bloody mucus is very important, because in this group of patients if the latter was found we could expect to find eggs of *S. japonicum* readily and quickly by direct examination of such material in about 90 per cent of the specimens. No further examination then is necessary. Second, direct smear examination of nonbloody mucous fecal material in the majority of patients particularly in those with light infections or after one or more courses of treatment, will yield relatively few positive results and only rare positives will occur which are negative by the water centrifugal sedimentation method. In our experience the latter method has confirmed all routine direct smear positives obtained from fecal specimens free of grossly visible bloody mucus. A reasonable technique therefore in a stool examination for suspected schistosomiasis is careful inspection of the specimen for bloody mucus, direct examination of the latter if present, and water centrifugal sedimentation if negative or not present.

3. *Zinc Sulfate Centrifugal Flotation:*² In the course of these studies approximately 600 patients with schistosomiasis had at least one examination by the foregoing method. The diagnosis of schistosomiasis was confirmed by the demonstration of eggs of *S. japonicum* at this hospital in 226 patients, but in no case was the diagnosis made by zinc sulfate. Ten specimens containing a large number of *S. japonicum* eggs were studied by the zinc sulfate method. In two specimens wrinkled masses of eggshells were found by zinc sulfate, but proper identification would have been impossible. If the speed of centrifugation is reduced below 1,000 r.p.m., distortion is less marked, but the eggs are still not suitable for routine identification. If the whole sediment obtained by water sedimentation is treated by the zinc sulfate method but centrifuged at less than 1,000 r.p.m., a large number of eggs will be brought to the surface. These are also poorly preserved, and this modification would have no merit since it would entail carrying out the procedure of two methods, the first of which alone gives a more satisfactory result. The standard zinc sulfate method routinely employed in patients suspected of having schistosomiasis is valuable in detecting protozoan cysts and larvae and eggs of some helminths but is of no value in the diagnosis of *S. japonicum* infections.

4. *Brine Flotation:* No patients with schistosomiasis japonica were discovered by this method which was applied at least once in all patients.

5. *Acid Ether:* Fifty specimens from different patients found to have positive results by water centrifugal sedimentation were examined by the acid-ether techniques of De Rivas³ and Weller and Dammin.⁴ The use of 40 per cent hydrochloric acid was superior to 5 per cent acetic acid, but both methods of acid ether yielded only sixteen positive results or an efficiency of only 0.32 compared to water centrifugal sedimentation (1.00). Ten specimens positive by

water centrifugal sedimentation but negative by acid ether were examined again by the acid-ether method with the addition of small amounts of quarter-master laundry detergent. Only one specimen became positive as a result of this modification, and it was not deemed worth while to explore this further. Acid ether alone was superior to direct smear examination of specimens without visible bloody mucus, but either method or both combined were far inferior to water centrifugal sedimentation alone and did not increase the yield of positives if performed in conjunction with the latter. Acid ether is not recommended as the procedure of choice for the diagnosis of schistosomiasis japonica infections.

6. *Comparison of All Methods in a Series of Fifty Consecutive Stools Positive for Eggs of S. japonicum*: The findings summarized in Table I bear out the data presented and discussed in the preceding paragraphs. One notes that in this series zinc sulfate and brine flotation failed to detect a single infection.

Examination by direct smear of bloody mucus present in eight specimens was positive in seven, and the remaining specimen was positive by water centrifugal sedimentation. The latter method alone would have missed three specimens positive by direct smear. Examination of feces from areas not containing grossly visible bloody mucus by direct smear detected nine (18 per cent) positives, all of which were also positive by water centrifugal sedimentation and four of which were positive by examination of mucus from the same specimen. Direct smear examination of fecal material with and without bloody material thus detected twelve (or 24 per cent) of the total patients with positive results in this series.

The acid-ether method permitted detection of sixteen positive results (32 per cent), all of which were also positive by water centrifugal sedimentation. Acid ether detected seven positives not discovered by direct smear of fecal specimens free of grossly visible bloody mucus. The one specimen of bloody mucus which was negative by direct smear was also negative by acid ether, and three positive bloody mucus specimens were negative by acid ether. Acid ether alone, therefore, would have discovered 32 per cent of the positives, and, if combined with direct smear of specimens with or without bloody mucus, the acid-ether method would have detected nineteen (or 38 per cent) of the total positives.

Water centrifugal sedimentation detected forty-seven (or 94 per cent) of the total positive results in this group of fifty patients; the remaining three having been found to have positive results by direct smear of blood mucus and negative by water centrifugal sedimentation.

Consideration of these data indicates that the most practical and efficient method of detection of eggs of *S. japonicum* in stool specimens consists of (1) careful visual examination of the entire gross specimen for the presence of mucus or bloody mucus and, if present, the latter is examined by direct smear and (2) water centrifugal sedimentation examination if mucus or bloody mucus is absent or negative by direct smear examination if present.

Number of Stool Examinations for Diagnosis and Discovery of Treatment Failures.—In the presence of active infection with *S. japonicum* several factors

TABLE I. RESULTS OF STOOL EXAMINATIONS FOR EGGS OF *S. JAPONICUM* BY VARIOUS METHODS IN FIFTY PROVED CASES

CASE	SMEAR BLOODY MUCUS	DIRECT SMEAR	ACID ETHER	CENTRIFU- GAL SEDI- MENTATION	ZINC SULFATE	BRINE FLOTATION
1		0	0	X	0	0
2	P X	X	X	X	0	0
3		X	X	X	0	0
4		0	0	X	0	0
5	P X	0	0	0	0	0
6		0	0	X	0	0
7		0	X	X	0	0
8		0	0	X	0	0
9	P X	X	X	X	0	0
10		0	X	X	0	0
11		0	0	X	0	0
12		0	0	X	0	0
13		0	X	X	0	0
14		0	0	X	0	0
15		0	X	X	0	0
16	P X	0	0	0	0	0
17		0	0	X	0	0
18	P O	0	0	X	0	0
19		0	0	X	0	0
20		0	0	X	0	0
21		0	0	X	0	0
22		0	0	X	0	0
23	P X	X	X	X	0	0
24		0	0	X	0	0
25		0	0	X	0	0
26	P X	0	0	0	0	0
27		X	X	X	0	0
28		0	0	X	0	0
29		X	X	X	0	0
30		X	X	X	0	0
31		0	0	X	0	0
32		0	0	X	0	0
33	P X	X	X	X	0	0
34		0	0	X	0	0
35		0	X	X	0	0
36		0	0	X	0	0
37		0	0	X	0	0
38		0	0	X	0	0
39		0	0	X	0	0
40		0	X	X	0	0
41		0	0	X	0	0
42		0	0	X	0	0
43		0	X	X	0	0
44		0	0	X	0	0
45		0	0	X	0	0
46		0	0	X	0	0
47		X	X	X	0	0
48		0	0	X	0	0
49		0	0	X	0	0
50		0	0	X	0	0
Total		9	16	47	0	0

P, present; X, positive; 0, negative.

other than the efficiency of the technique of stool examination will have an influence on whether or not eggs are found in the feces. These are the stage of the disease, the degree and extent of intestinal involvement, whether specific treatment has been given, and, if so, the drug and total amount, the time interval following completion of treatment when stool examinations are be-

gun, and the total number of stools examined. These factors will be discussed briefly.

The acute stage of the disease will not ordinarily be seen in this country. However, the number of stool examinations necessary to establish the diagnosis during the acute stage of the disease experienced overseas is of interest. Of fifty patients with positive stools overseas subsequently found to have positive stools at this hospital, results in twenty-two were found positive overseas on the first examination, seventeen in from two to five examinations, ten in from six to ten, one was positive in from eleven to twenty, and one in more than twenty stool examinations. The average number of stool examinations necessary to demonstrate eggs of *S. japonicum* during the acute stage of the disease overseas in this series of patients who were examined within from two to four months after first possible exposure was 3.6. The method of stool examination at overseas hospitals was not specified but probably consisted of direct smear and some form of water sedimentation. Stool examinations in these same patients were all found positive here after prior treatment. In nine patients results were positive in the first five examinations, ten in from six to ten, eleven in from eleven to fifteen, six in from sixteen to twenty, seven in from twenty-one to twenty-five, and seven in from twenty-six to thirty-five stool examinations. The average number of stools examined before eggs were found was fifteen. It is apparent then that eggs are found more readily and with fewer stool examinations during the acute stage of the disease than later after treatment has been given.

In order to determine whether eggs once present appear regularly thereafter, stools from patients found positive were examined daily until the next positive result was found. Of fifty such patients, results in thirty-seven were positive on two consecutive days, five again on the third consecutive examination, two on the fourth, two on the eighth, and one each on the fourteenth, nineteenth, twentieth, and twenty-second consecutive examinations. This indicates the irregularity with which eggs of *S. japonicum* may appear in the stools, since from one to twenty-two days or more may elapse between two positives and emphasizes the necessity for repeated examinations before the diagnosis can be excluded reasonably.

Success in discovering treatment failures is governed largely by consideration of the type and amount of treatment, the total number of stools examined, and when, after treatment, such examinations are begun and how long continued. Stool examinations during the first three or four weeks immediately after treatment are of no significance since eggs already laid prior to treatment may require this length of time to leave the body and do not indicate treatment failure.⁵ As a rule treatment failures after fuadin are discovered at shorter intervals (from five to seven weeks) than after tartar emetic (from eight to twelve weeks following completion of treatment). Fifty of eight stool examinations at Moore General Hospital required an average interval of sixty days beginning thirty days after completion of treatment before eggs were discovered. It is therefore suggested that stool exam-

inations be started thirty days after completion of treatment and such examinations be continued (three specimens per week) for the next eight to ten weeks. Since the majority of patients with schistosomiasis japonica will require one or more courses of treatment, consideration of these factors is important in discovering treatment failures. Treatment failures can only be discovered by repeated stool examinations during a relatively long period of time. In evaluating suspected schistosomiasis in patients with a definite history of exposure in an endemic area and suggestive clinical findings, from twenty to thirty stool examinations in from four to six weeks may be required to establish or exclude the presence of eggs in the stools. Persistently negative stools in some patients do not preclude the possibility of active infection.

Parasites Other Than S. japonicum Discovered by Water Centrifugal Sedimentation.—In the course of numerous examinations by the previously mentioned method of stool specimens from patients with suspected or proved schistosomiasis, other parasites were frequently discovered. In 100 patients found to have *S. japonicum* eggs in the stool as a result of one or more examinations by water centrifugal sedimentation, 4 per cent of the patients were found to have cysts of *Endamoeba histolytica* in the sediment, 18 per cent, 20 per cent, and 4 per cent had eggs of hookworm, *Trichuris trichiuria* and *Ascaris lumbricoides*, respectively, and 2 per cent had larvae of *Strongyloides stercoralis*. A single zinc sulfate examination in each of the same patients showed 4 per cent, 9 per cent, 4 per cent, 1 per cent, and 2 per cent to have these parasites in the order given. A single brine flotation in these patients detected 13 per cent to have hookworm infections, and six and 2 per cent had *Trichuris* and *Ascaris*, respectively.

No comparison of the relative efficiency of these methods in detecting protozoa or helminths is intended. Each patient with stools positive for *S. japonicum* had on the average fifteen water sedimentation examinations before the diagnosis was established and at least thirty additional examinations after treatment, whereas zinc sulfate and brine flotation were done only once except to evaluate treatment for hookworm or amebiasis. However, it is important to note that in the course of many water centrifugal sedimentation examinations for the diagnosis or evaluation of therapy in schistosomiasis other pathogenic protozoa or helminths may be found, which may or may not be found also in a single zinc sulfate, brine flotation, or direct smear examination.

SUMMARY AND CONCLUSIONS

1. Water centrifugal sedimentation is a simple rapid method of stool examination for detecting eggs of *S. japonicum* in the stools of infected patients.

2. This method requires no chemical reagents, and only a minimum amount of standard laboratory equipment is necessary.

3. In our experience it was more efficient than direct smear examination of stool material free of grossly visible bloody mucus or examination by acid-ether, zinc sulfate, or brine flotation. Viability of eggs found after treatment can be determined.

4. Direct smear examination of bloody mucus present in some stools revealed a high percentage of positives for *S. japonicum* in such material. Occasionally trophozoites of *E. histolytica* also were found in this material.

5. Water centrifugal sedimentation done repeatedly in the course of the diagnosis of suspected schistosomiasis, or for evaluation of specific therapy, will frequently detect the presence of other protozoa and helminths in addition to *S. japonicum*.

6. In our experience the stool method of choice in the study of patients with schistosomiasis japonica is as follows:

(a) Careful visual examination of the gross stool for the presence of bloody mucous material. Direct smear examination of this material if present.

(b) Water centrifugal sedimentation of all specimens not containing grossly visible bloody mucus and specimens negative by direct smear of bloody mucus if present.

(c) Routine zinc sulfate and brine flotation for the detection of protozoa and helminths other than *S. japonicum* which may or may not be found by water centrifugal sedimentation and which may be present in a high per cent of patients under observation for schistosomiasis japonica.

7. From twenty to thirty stool examinations during a period of from four to six weeks were required to detect light asymptomatic infections in a group of patients exposed on Leyte. Eggs had not been detected previously in any of these individuals, and none had received treatment.

8. Following treatment stool examinations should be begun thirty days after completion of treatment and continued for from eight to ten weeks (three stools weekly). Most treatment failures are discovered from five to twelve weeks after completion of therapy with fuadin or tartar emetic.

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A PRESUMPTIVE MEDIUM FOR DIFFERENTIATING PARACOLON FROM SALMONELLA CULTURES

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WITH the increasing attention that is being given to the bacteriologic characteristics and pathogenic significance of the paracolon bacilli, the need has arisen for prompt and effective tests by which they may be recognized. Essentially, the paracolon group is characterized by the single property of fermenting lactose slowly. This is not a satisfactory characteristic for use in diagnostic laboratory work because, as Monteverde¹ and others have well shown, it is very slow to appear, irregular even with the same pure culture, and in general it is an unpredictable reaction. The advantage of the prompt reporting of identifications, if the physician is to be guided in treatment by knowledge of the type of bacterial infection existing in the patient, makes less time-consuming tests very necessary.

Since Russell,² in 1911, advocated the first double sugar agar medium for the differentiation of gram-negative enteric bacilli on the basis of lactose and glucose fermentation, many workers have proposed similar one-tube test media. Among the more recent media are Hajna's,³ Sulkin and Willett's,⁴ Felsenfeld and Young's,⁵ Krumwiede and Kohn's,⁶ Kligler's,⁷ and Friewer and Shaughnessy's.⁸ Felsenfeld proposed using his medium not as a screen test but to confirm the reactions of organisms suspected of being *Salmonella* or *Shigella* on the basis of a previously performed test on Friewer and Shaughnessy's medium. The successive use of two media, the second of which had to be observed for four days, did not accomplish the objective of rapid separation of the *Salmonella* and paracolon groups.

The problem of prompt recognition of paracolon cultures was approached by comparing the fermentation reactions of over 250 paracolon strains with the reactions of the strains of *Salmonella* in the standard collection of *Salmonella* type cultures. There were various tests in which paracolon cultures gave positive results while *Salmonella* were negative. By combining these test substances into a single medium, a presumptive test medium was secured in which a positive reaction eliminated the possibility that the test organism belonged to the *Salmonella* group. The new multiple sugar medium was intended for use as a preliminary test with which to distinguish paracolon cultures from *Salmonella*, replacing lactose broth which had proved too slow and uncertain. For convenience this medium was called AASS, from the initials of the carbohydrates used in it.

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The composition of the AASS medium was: 0.5 per cent adonitol, 0.5 per cent aesculin, 0.5 per cent salicin, and 0.5 per cent sucrose dissolved in purple broth base.* Two indicators were employed: (a) bromeresol purple, 0.015 Gm. per liter as present in purple broth base, and (b) ferric ammonium citrate brown scales, 0.05 Gm. per liter. The carbohydrates dissolved in the broth were tubed in from 2 to 3 c.c. amounts and sterilized in the usual manner. The AASS broth was also tested using gas vials, and it gave the same reactions within the same limits of time as the medium without the vials. Hence, gas production could also be observed in this medium if desired.

None of the four carbohydrates was fermented by *Salmonella*, whereas one or more of them was usually fermented by the paracolon organisms. The medium became yellow if adonitol, salicin, or sucrose was fermented. If aesculin was fermented the medium blackened. In addition to the fermentation of the sugars, Kovaacs' test for indole production was superimposed on these cultures, thus making it possible to detect many paracolon strains which were indole positive but did not ferment the carbohydrates. In this connection it may be pointed out that these indole tests were made on cultures which failed to ferment any of the four carbohydrates which were present. As a matter of interest it can be recorded that indole tests were also made on the cultures which showed positive fermentation reactions, with results that agreed with the known indole reaction of the culture obtained in all cases. Over 250 paracolon strains were tested in the AASS medium, and it was found to be about 80 per cent efficient in indicating that a culture belonged to the paracolon group. This result was obtained with cultures incubated for twenty-four hours at 37° C.

The paracolou group could be separated into three types on the basis of biochemical reactions in the AASS medium: (1) those which fermented one or more of the carbohydrates in twenty-four hours, (2) those which did not ferment any of the sugars but were indole positive in twenty-four hours, and (3) those which did not ferment the sugars and which were indole negative in twenty-four hours. Forty-four per cent of the 257 cultures tested fermented at least one of the sugars in twenty-four hours. Some of these tubes were yellow indicating that adonitol, salicin, or sucrose had been fermented, while others were black indicating fermentation of aesculin. Thirty-five per cent were fermentation negative and indole positive. The indole tests checked with the results obtained by Kovaacs' test in the usual 1.0 per cent tryptone broth. Twenty-one per cent of the paracolon strains were negative in both tests. AASS cultures of this group were observed for seven days, and only eleven of the strains were slow fermenters of the mixture of carbohydrates. Among this group of fifty-five cultures, twenty-two gave evidence of ability to ferment lactose when grown for twenty-four hours at 37° C. on a heavily inoculated slant of 10 per cent lactose in purple agar base.* This comparatively aerobic method of testing for ability to ferment lactose was found to be superior in many instances to any other broth semisolid or solid stab medium which was tested.

*Difco Laboratories, Inc., Detroit, Mich.

The AASS medium can be used in accordance with the following suggested routine: Possible paracolon cultures are inoculated into the AASS medium and onto slants of 10 per cent lactose agar containing bromeresol purple indicator. Incubate for twenty-four hours at 37° C. Positive reactions in AASS will be considered to be acidification, blackening, or a positive Kovacs' indole test. The 10 per cent lactose slant will be read positive when (a) the indicator in the agar is changed or (b) when daughter colonies showing the acid color of the indicator are observed arising on the growth on the slant. The identification of the paracolon group is indicated when either or both of these two tests is positive in twenty-four hours.

Proteus cultures may give a positive AASS test, as may *Shigella*. The latter can be excluded by the slide agglutination test as developed by Weil and associates.⁹ The recognition of *Proteus* cultures as a group is best made by the test for hydrolysis of urea as proposed by Rustigian and Stuart.¹⁰ Further investigations are needed in order to devise means for the prompt classification of the group of strains which were negative in AASS and on the 10 per cent lactose slant. At present the complete study of such strains is the only known way to evaluate them correctly, and this study is both cumbersome and time consuming.

AASS medium has the psychologic advantage of providing a positive reaction rather than the negative test results which all too often have been the sole basis for the classification of cultures in the paracolon group. The AASS medium provides a rapid means of recognizing paracolon cultures and hastens diagnostic work by eliminating the long uncertain wait for slow lactose fermentation to occur.

SUMMARY

1. A new multiple-carbohydrate medium was devised as a means of differentiating paracolon bacilli as a group from *Salmonella* organisms. The medium contained 0.5 per cent each of adonitol, aesculin, salicin, and sucrose. From the initial letters of the four component carbohydrates the name AASS medium was invented.

2. The AASS medium was a rapid means of differentiating paracolon from *Salmonella* cultures on the basis of fermentation of one or more of four carbohydrates which are utilized by paracolon bacilli but not by *Salmonella*.

3. Ability to produce indole was demonstrated accurately in the AASS medium and was an additional differential characteristic of great value.

4. Eighty per cent of the paracolon strains were detected within twenty-four hours by the use of this medium.

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BOOK REVIEWS

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THE ELIMINATION OF HUMAN REACTION-PRODUCING MATERIAL FROM HORSE-DERIVED ANTISERUMS: AN ELECTRICAL METHOD

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INTRODUCTION

IMMUNE serum therapy in man has been associated with certain serum reactions^{1, 2} that have done much to discourage its use in the patient.

The causes for these reactions have not been completely eliminated from sera by purification and concentration. These efforts have ranged from heating to complex chemical protein fractionations.^{3, 4} The aim, in general, has been to eliminate all the immunologically inert protein from sera in the hope that the reaction-producing substances would go with them. The remaining antibody proteins are concentrated to increase the potency of the antibody per unit of volume. A smaller dose is then possible.

More recently the possibility that bacterial contaminants may produce pyrogen for man^{5, 6} has spurred the preparation of immune sera under sterile conditions.

In our laboratory we have purified antibacterial sera by eliminating an acid fraction of protein. The antibody-bearing globulin has been collected for concentration by water precipitation.⁷ This method has been improved upon from time to time,^{8, 9} but the products so obtained still cause untoward reactions upon intravenous injection in man.

The electrical method to be described herein was used chiefly to reprocess pneumococcic antiserum preparations that had been shown to contain immediate reaction-producing material by clinical test. They had been purified and concentrated with the water precipitation method.⁷ After the electrical reprocess they were again tested clinically to determine to what extent the reaction-producing material had been eliminated.

APPARATUS

The apparatus (Fig. 1) consists of six electrodes, each made up of a zinc jacket filled with tap water and with a platinum wire disposed in its center. The latter is insulated from the zinc jacket by a rubber plug at the top and a rubber washer at the bottom.

Cellulose casings,* with a diameter and length suitable to the size of the apparatus, are cut and one end is wet in water. While wet, this end is constricted manually by folding and is sealed by wrapping one or two rubber bands tightly around it.

From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York City.

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*Bought from the Visking Cellulose Casing Corp., Chicago, Ill.

For best results the casings must be large enough to contain one-seventh the volume of diluted serum to be processed. When they have been filled with this volume of tap water, the zinc jacket electrodes are immersed in each one. The semipermeable membranes are supported in place by round metal frames to which they are secured. These are fastened by means of screws to a glass top which fits snugly over the vat containing the serum. The zinc jacket electrodes are also supported by the glass top through holes cut in it, and each is suspended in the center of the cup formed by the cellulose casing membrane. These metal electrodes are immersed in the water contained in the membranes to at least two-thirds of their length. Two electrically driven stirrers inserted through holes in the glass top complete the apparatus.

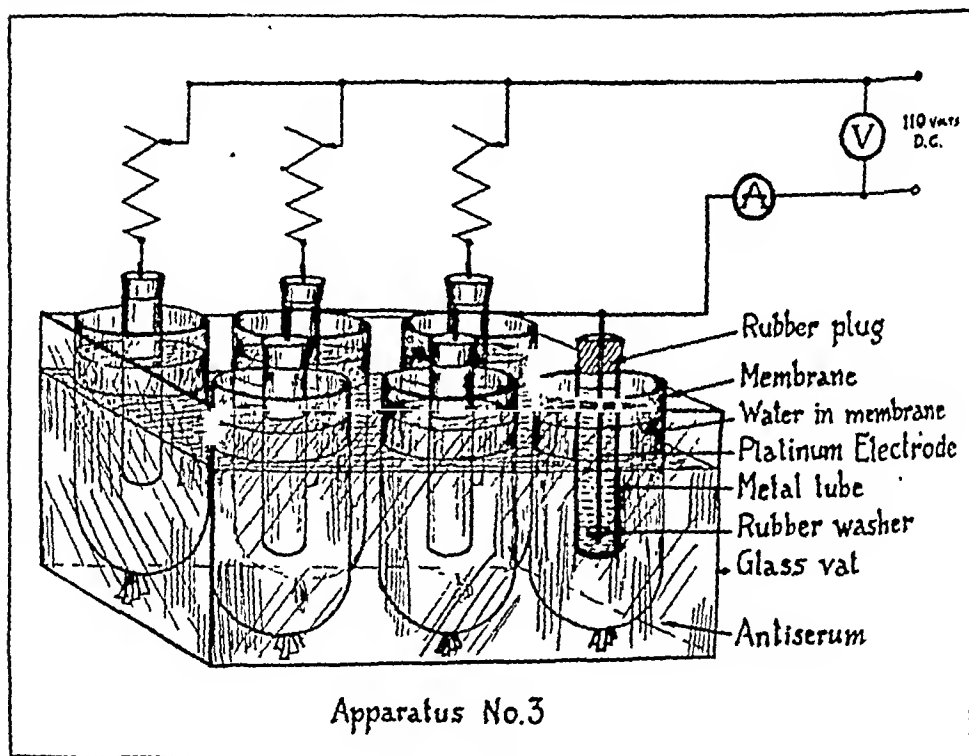


FIG. 1.

PROCEDURE

The serum* to be processed is adjusted to pH 5.1 with normal acetic acid. It is diluted in the proportion of one volume to three and one-half volumes of distilled water freed of carbon dioxide gas by boiling. Solid sodium chloride is added to give a final concentration of one-half of one per cent. The material is poured into the vat of the apparatus. A 110-volt direct current is turned on and by means of series resistances is adjusted to 60 ma. with an electromotive force of approximately 90 volts.

*Our concentrated antibacterial sera contain about 13 mg. N/Gm. of serum.

The pH value is determined at intervals. When it reaches pH 7.2 the electrical process is stopped, and the serum is removed from the vat. At this point all the globulin proteins are precipitated. The pH is adjusted to 5.1 with normal acetic acid; the serum is placed in cellophane casings 6 inches in diameter and 24 inches long ($1\frac{1}{2}$ liters per casing) and dialyzed for six days in a running water bath at 15° C. with moderate agitation. At the end of six days the reaction of the dialysate is between pH 5.7 and 6.3. The reaction is brought to pH 5.1 with normal acetic acid. Saturated ammonium sulfate solution is added slowly and with constant stirring to 30 per cent saturation. The pH is adjusted to 5.9 with normal sodium hydroxide, and the serum is left at room temperature overnight. The clear supernatant fluid is siphoned off, and the precipitate is centrifuged. The supernatant fluid is added to the material siphoned off, and the precipitate is discarded. Saturated ammonium sulfate solution is again added to 50 per cent saturation. After one hour at room temperature the solution is filtered through hardened paper. The filtrate is discarded, and the precipitate is thoroughly dried by pressing between pads of absorbent paper. The precipitate is then dialyzed until free of sulfate ion. If the nitrogen content of the dialysate is higher than desired, it is diluted to a suitable value with distilled water. Solid sodium chloride to a concentration of 1 per cent and suitable preservatives are added. The pH is adjusted to 7.4 with normal sodium hydroxide, and the solution is left in the cold overnight.

The treated serum is clarified by centrifugation. It is then filtered through a Berkefeld stone to sterilize it, and its potency is determined!

CLINICAL RESULTS

During the development of the chemical procedure following the electrical process, it became evident that when the sera were not dialyzed or were dialyzed insufficiently after the electrical preparation they did not lose reaction material at all or they lost it in proportion to the degree of completion of dialysis. These data are presented in Table I.

Type 1, Preparation 107, was clinically free from reactions before and after reconcentration. However, it had been found to contain toxic material for the rabbit which was lost after the electrical preparation and six and a half days of dialysis. Type 8, Preparation 23, had been deprived of the important last day of dialysis.* Upon clinical trial this preparation was still found to contain considerable reactive material.

The remaining preparations recorded in Table I had been dialyzed for four days or less following the electrical treatment. They show little or no improvement in the nature of the reactions caused in man or in the rabbit.

That dialysis of sera without electrical preparation does not eliminate reaction-producing material from them was proved in our laboratory over a period of many years. Serum lots, prepared regularly,^{7, 9} that had been shown to contain reactive material by clinical trial were reprocessed by different chemical procedures in an effort to develop one that would eliminate this material.

*On this day more reactive material is eliminated than on earlier days, because it is not hampered by other impurities with faster rates of dialysis.

TABLE I. REACTION-PRODUCING MATERIAL, MADE DIALYZABLE BY ELECTRICAL PREPARATION, NOT ELIMINATED COMPLETELY BY LESS THAN SIX DAYS OF DIALYSIS

BY ELECTRICAL PREPARATION, NOT ELIMINATED COMPLETELY BY LESS THAN SIX DAYS OF DIALYSIS

RECONCENTRATED PNEUMOCOCCIC ANTISERUM		POTENCY/C.C. (MOUSE UNITS)		NITROGEN VALUES (MG. N/GM. OF SERUM)		RABBIT TOXICITY TEST ^{11, 12} (° F.)				RESULTS OF CLINICAL TRIAL*		DAYS OF DIALYSIS AFTER ELECTRICAL PREPARA- TION		
		ANTISERUM		ANTISERUM		ANTISERUM				ANTISERUM BEFORE RECONCENTRATION			ANTISERUM AFTER RECONCENTRATION	
		BEFORE RECONCEN- TRATION	AFTER RECONCEN- TRATION	BEFORE RECONCEN- TRATION	AFTER RECONCEN- TRATION	TEMPERA- TURE RISE BEFORE TRATION	TEMPERA- TURE RISE AFTER RECONCEN- TRATION	TEMPERA- TURE RISE BEFORE TRATION	TEMPERA- TURE RISE AFTER RECONCEN- TRATION	Acceptable	Acceptable			
TYPE	PREPARA- TION NO.	5058	4012	14.53	13.53	1.9	0.3	1.3	17 treated	%	7 treated	%		
8	23	3000	3000	13.7	13.4	Not done	1.3		No reactions	33	No reactions	52		
18	17	3700	3493	10.9	9.05	1.5	1.4		Dyspnea	53	Dyspnea	53		
	19	6212	9312	13.0	11.7	2.4	2.5		Chill	6	Chills	14		
	34	3756	2390	14.55	9.95	2.3	1.9		Urticaria	6	Urticaria	0		
									Nausea and vomiting	14	Vomiting	14		
									Blood pressure drop	6	Blood pressure drop	0		
5									Reactions		Not done; toxic for rabbits	4.0		
									Reactions		Not done; toxic for rabbits	3.5		
									Reactions		Not done; toxic for rabbits	3.0		

8	39	2400	3100	12.2	15.15	2.1	0.9	Not done; toxic for rabbits	Gave chill in one case treated	2.5
8	40	1514	3078	11.3	12.9	2.4	1.9	Reactions	Not done; toxic for rabbits	2.5
8	44	2307	1983	13.2	11.35	2.3	1.2	Reactions	Not done; toxic for rabbits	2.5
5	23	2817	2970	12.7	13.85	1.9	2.5	12 treated No reactions Dyspnea Chills Urticaria Nausea Headache	3 treated No reactions Dyspnea Chills Urticaria Temperature rise Vomiting Pain in chest	1.5
1	79	3000	3000	11.75	14.05	2.3	1.6	Preparations pooled for this common original had caused shock, chills, dyspnea, and urticaria	2 treated No reactions Dyspnea Chills Urticaria	0
1	80	6000	6000		13.35		1.8			
									3 treated No reactions Dyspnea Chills Urticaria	2

*Most of these preparations had been tested clinically before the rabbit test had been adopted as the basis for determining whether or not a serum was to be tested clinically.

TABLE II. REACTION-PRODUCING MATERIAL, MADE DIALYZABLE BY ELECTRICAL PREPARATION, ELIMINATED BY SIX DAYS OF DIALYSIS

PNEUMO-COCCIC ANTISERUM		POTENCY/C.C. (MOUSE UNITS)		NITROGEN VALUES (MG. N/GM. OF SERUM)		RABBIT TOXICITY TEST ^{11, 12} (° F.)		RESULTS OF CLINICAL TRIAL	
TYPE	PREPARATION NO.	ANTISERUM		ANTISERUM		ANTISERUM		ANTISERUM	
		BEFORE RECONCENTRATION	AFTER RECONCENTRATION	BEFORE RECONCENTRATION	AFTER RECONCENTRATION	TEMPERATURE RISE BEFORE RECONCENTRATION	TEMPERATURE RISE AFTER RECONCENTRATION	BEFORE RECONCENTRATION	AFTER RECONCENTRATION
2	83	4387	6150	14.35	10.9	1.8	0.2	All had caused immediate serum reactions	Acceptable
1	88	5050	5600	12.85	10.7	0.8	0.1		Acceptable
5	31	5010	5850	12.75	15.45	2.0	1.0		Acceptable
4	38	4775	3750	12.85	10.8	1.7	0.2		Acceptable
5	35	2953	3680	13.3	13.1	2.2	1.0		Acceptable
8	45	2990	2490	12.85	12.85	2.1	0.3		Acceptable
7	34	1200	2167	11.4	15.25	2.0	0.8		No clinical test report available
4	36	1750	2100	12.75	10.25	2.7	0.5		
2	92	1680	1430	14.65	12.0	1.7	0.7		
1	98	2612	2087	12.6	13.7	1.1	0.5		
8	43	3423	3553	13.5	12.9	3.0	0.4		
18	18	8800	6280	13.2	11.75	1.5	0.5		

*An antiserum that causes an average temperature rise higher than 1° F. in three rabbits^{11, 12} may cause reactions if injected intravenously in man. When the rabbit temperature rise is 1° F. or less the antiserum is considered unlikely to cause reactions in man.

†An "acceptable" serum caused either no reactions upon intravenous injection in man or the reactions were of such a mild nature that the serum was considered satisfactory for distribution.

TABLE III. ELECTRICAL METHOD COMPARED TO THREE OTHER METHODS IN ELIMINATION OF IMMEDIATE REACTION-PRODUCING MATERIAL FROM A COMMON ORIGINAL ANTISERUM

ANTISERUM		METHOD OF PREPARATION	RESULTS OF CLINICAL TRIAL	RABBIT TOXICITY TEST ^{11, 12} (° F.)
TYPE	PREPARATION NO.			
Common original,				
5	22	Prepared with regular method ^{5, 9} ; this serum had been dialyzed for 6 days	4 treated 3, chills 1, chilly sensation 2, temperature rise 1, vomiting	2.0
5	32	Used $\frac{1}{3}$ of common original; serum heated to 56° C. for $\frac{1}{2}$ hour and adsorbed with kaolin according to technique of Goodner and associates ¹¹ ; globulin, precipitated by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$, dialyzed for 6 days before use	Not tested clinically because too low in potency and because the proteins had become irreversibly insoluble, probably due to heating	0.7
5	33	Used $\frac{1}{3}$ of common original; fraction of protein precipitated up to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ discarded; fraction precipitated up to 50% $(\text{NH}_4)_2\text{SO}_4$ saturation dialyzed for 6 days before use	Not tested clinically because toxic for rabbits	1.3
5	31	Used $\frac{1}{3}$ of common original; Prepared by electrical method with 6 days of dialysis	3 treated 3, no reactions	1.0

After each effort the serum was dialyzed for six days. Upon clinical retest these sera still caused immediate serum reactions in man.

The serum preparations recorded in Table II were made from regularly prepared lots^{7, 9} that had been shown to contain reactive material for man upon clinical trial. Furthermore, all but one gave more than the acceptable temperature rise in the rabbit test for pyrogen. Each was processed electrically and then dialyzed for six days. Upon test it was found that all had been cleared of significant pyrogenic material for the rabbit, and those preparations that it was possible to test clinically had lost the power to cause immediate serum reactions in man.

In Table III is presented the comparison in power to eliminate human and rabbit reaction material from pneumonia horse sera by the electrical procedure and six days of dialysis and three other methods of preparation also followed by six days of dialysis. The common original used for these preparations was an antiserum which had been prepared with the water precipitation method^{7, 9} and which had caused immediate serum reactions in man when tested clinically. The electrical method eliminated the reactive material more effectively.

RESULTS OF THIS METHOD WITH OTHER BIOLOGICALS

Other biologic products have been reprocessed with this method to improve them.¹⁰ In Table IV are shown the results of its adaptation to the reconcentration of antimeningococcus sera prepared by the water precipitation method.^{7, 9} The potency per unit of protein nitrogen was increased, allowing smaller injections and thus rendering the preparations more suitable for human use.

In other experiments antisera of latinal globulin reprocessed by this method was tested by Dr. Samuel Karelitz in patients at the Willard Parker Hospital. It was found that the incidence of delayed serum sickness in the sixty-four patients treated with this material was about 38 per cent. More significant, however, was the unusually mild nature of these reactions as compared with those caused by regular material.*

TABLE IV. MENINGOCOCCUS ANTISERUM MADE MORE POTENT PER UNIT OF PROTEIN NITROGEN BY ELECTRICAL PROCESS

IRREGULARLY PREPARED, ⁹ PREPARATION NO.	ELECTRICAL RECONCENTRATION PREPARATION NO.	AGGLUTINATION TEST*				OTHER TESTS*			MG. N/GM. OF SERUM
		TYPE AND ANTIGEN NO. USED				AGAR PLATE PRECIPITATION	S-I PRE- CIPITATION	MOUSE PROTECTIVE RATIO	
		I	I	III	II				
		331	1027	302	36				
77		2.0	2.0	2.0	2.0	+++	1-512	336%	11.7
	77S	2.0	2.0	2.5	16.0	+++++	1-2048	653%	11.65
79		1.5	1.5	2.0	8.0	+++	1-225	225%	9.75
	79S	2.0	1.0	0.6	8.0	+++	1-512	157%	6.55
72		1.2	2.0	2.0	16.0	+++	1-1024	184%	11.3
	72S	1.2	2.0	2.0	16.0	+++	1-1024	186%	6.8
N. I. H. Control		1.0	1.0	1.0	1.0	+++	1-512	100%	-

*The potencies were determined by Mrs. C. R. Falk of the New York City Department of Health.

*Personal correspondence from Dr. Samuel Karelitz.

DISCUSSION

Whether the reaction-producing material is adsorbed on the surface or whether it is a more integral part of the large molecular aggregate which is the antibody entity, it is freed by the electrical process. Whether it is of organic or of inorganic origin, it is in a form, or it is changed to a form, that is eliminated or inactivated by simple dialysis.

SUMMARY

An electrical process is described which, when followed by six days of dialysis, has eliminated from purified and concentrated horse-derived antiserum substances which had caused immediate serum reactions when injected intravenously in man.

In most lots tested it was possible to remove reaction-producing material by this method without lowering the antibody potency per unit of protein nitrogen. Indeed, in the majority of lots the potency was increased.

The author is indebted to Dr. Wheelan D. Sutliff and Miss Annabel W. Walter of the Pneumonia Control Division, for their valuable cooperation in carrying out potency determinations and rabbit tests and in supplying results of clinical trial as indications of the presence or absence of reaction-producing material.

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A STUDY OF ABNORMAL T WAVES IN PATIENTS PRESENTING NO EVIDENCE OF ORGANIC HEART DISEASE

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EVER since Einthoven introduced the electrocardiogram, the interpretation and significance of the T wave has been a matter of much discussion. We still do not know the exact part the T wave plays in the electrodynamics of the cardiac cycle. The prevalent opinion is that the T wave represents the recession of the electrical impulse in the ventricles. According to Mines, as quoted by Wilson and Finch,¹ the form of the T wave depends on the order in which various regions of the ventricular muscle complete their electric activity. If the apex of the left ventricle returns to the resting state in advance of the base of the right ventricle, T is positive; if the reverse occurs, the T wave is negative.

The normal T wave is too well known to bear description at this time. Low voltage, biphasic, or inverted T waves in the first, second, or precordial leads are considered to be abnormal. In children or in pregnant women these aberrations in the T wave in the fourth lead are normal. The present paper does not include patients of this type.

The present study was made among a group of soldiers at a large Army hospital. Most of the subjects were from 20 to 40 years of age; some were older, and a few young women were included. The patients varied in body build in all extremes. Some presented numerous complaints suggestive of the so-called functional cardiac disorders; others appeared for examination only as a routine procedure. They presented two factors in common: (1) abnormality of the T waves in the electrocardiogram (there were no associated R-T or S-T segment changes); (2) none presented any other evidence of organic heart disease, as could be discovered by complete physical examination in which auscultation of the heart was performed in the upright, recumbent, and left lateral positions prior to and following exercise (hopping on each leg fifty times). Roentgen and fluoroscopic heart studies and sedimentation rate determinations were within the normal range except for the sedimentation rate in Patient 25 (Table I) whose case history will be taken up in greater detail later.

The electrocardiograms were obtained in the recumbent position and were taken at frequent intervals both before and after exercise (Figs. 1 to 5).

From November, 1943, to November, 1945, 5,520 electrocardiograms were taken at this hospital on 4,810 patients. Of these, fifty-one, or 1.0 per cent, showed significant T-wave changes, twenty-three predominantly in the chest lead (CR₄) (Table II), and twenty-eight showed the deviations in the limb leads (Table I) in individuals without any other evidence of heart disease.

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TABLE I. LIMB LEADS

PATIENT	AGE	SEX	BODY BUILD	DIAGNOSIS	ABNORMAL T WAVES	EFFECT OF EXERCISE
1	22	M		Psychoneurosis	II, III, low voltage	Normal
2	26	M		Psychoneurosis	II, low voltage	Normal
3	40	M	Medium	Psychoneurosis	II, low voltage	Normal
4	24	M	Medium	Psychoneurosis	II, isoelectric	Normal
5	26	M	Slender	Psychoneurosis (?)	I, low voltage	Normal
6	25	M	Medium	Psychoneurosis	I, II, low voltage	Normal
7	25	M		Transient hypertension	I, II, III, low voltage	Normal
8	30	M	Medium	No disease	II, inverted	Low voltage
9	29	M	Medium	Arthritis of feet (?)	II, inverted	Low voltage
10	35	M		Psychoneurosis	II, isoelectric	Normal
11	19	M	Medium	Psychoneurosis	II, inverted	Normal
12	29	M		Psychoneurosis; nontoxic adenoma of thyroid	II, isoelectric	Normal
13	36	M	Husky	Postinfluenzal asthenia (?)	II, III, inverted; II, low voltage (two days later)	None
14	32	M		Psychoneurosis	II, III, inverted	None
15	20	M	Medium	No disease	II, III, inverted	None
16	23	M	Slender	Psychoneurosis	II, III, inverted	Normal
17	23	M	Medium	No disease	II, III, inverted (normal six days later)	II, III, normal; P-R, .28 second (see Fig. 8)
18	24	M	Husky	Psychoneurosis	II, inverted (normal three weeks later)	Normal
19	47	M	Husky	Transient intestinal obstruction	II, biphasic; III, inverted	Normal
20	27	M	Medium	Fracture (leg)	II, III, inverted	None
21	22	M	Medium	Rheumatoid arthritis (?)	II, isoelectric; III, inverted (normal three days later)	Normal
22	29	M	Medium	Psychoneurosis	II, low voltage (normal two days later)	Normal
23	37	M	Medium	Psychoneurosis	I, II, biphasic	Normal
24	26	M	Medium	Psychoneurosis (?)	II, inverted	Normal
25	29	M	Husky	Syphilis; psychoneurosis	I, transient inversion; II, III, inverted (see Fig. 4)	None
26	30	M	Medium	Psychoneurosis	II, III, inverted	None
27	23	M	Medium	Rheumatoid arthritis (?)	I, isoelectric; II, low voltage; I, low voltage; II, normal ten days later	Not performed
28	33	M	Husky	Psychoneurosis	II, isoelectric	Normal

TABLE II. LEAD IV (CR₄)

PATIENT	AGE	SEX	BODY BUILD	DIAGNOSIS	T WAVES	EFFECT OF EXERCISE
1	29	M	Medium	Psychoneurosis	IV, inverted	IV, upright
2	19	M		Arthritis (not rheumatic fever); psycho-neurosis	IV, isoelectric	IV, upright
3	20	M	Slender	Psychoneurosis	IV, inverted	IV, upright
4	37	M		Psychoneurosis	IV, biphasic	IV, upright
5	32	F	Medium	Psychoneurosis	IV, inverted	IV, upright
6	30	M	Husky	Psychoneurosis	IV, isoelectric	IV, upright
7	26	F	Slender	Psychoneurosis	IV, inverted	IV, upright
8	35	M	Medium	Psychoneurosis	IV, biphasic	IV, upright
9	22	M	Slender	Unknown	IV, inverted	No change
10	35	M		Psychoneurosis	IV, inverted	IV, upright
11	23	M	Medium	Psychoneurosis	IV, biphasic	IV, upright
12	27	M		Schizophrenia	IV, inverted	IV, upright
13	39	M	Medium	Tracheitis	IV, inverted	IV, upright
14	41	M	Medium	Peptic ulcer	IV, inverted	IV, upright
15	31	M	Medium	Bronchitis	IV, inverted	IV, upright
16	33	M	Medium	Meningitis	IV, low voltage	IV, voltage higher
17	42	M		Herniorrhaphy	IV, biphasic	IV, upright
18	48	M	Husky	Nasal polyps and sinusitis	IV, biphasic	IV, upright
19	32	F	Medium	Extrasystoles	IV, isoelectric	IV, upright
20	36	F	Medium	No disease	IV, inverted	IV, upright
21	21	M		No disease	IV, inverted	IV, upright
22	20	M	Medium	No disease	IV, inverted	IV, upright
23	49	M	Slender	No disease	IV, biphasic	IV, upright

ANALYSIS OF RESULTS

Age and Sex.—The age of our patients varied between 19 and 47, the average being 29.9 years. All but four of the group were men.

Body Constitution.—A record of the body constitution was not made in all instances. In forty patients where a notation was made, eight were of the stocky broad-chested type, six belonged to the tall slender group, while the largest majority (twenty-six) were of medium build.

Diagnosis.—The greatest number of our patients was in the psychoneurotic group. Twenty-nine, or 57 per cent, were included under this heading (one individual suffered with schizophrenia). In addition, one patient had psychoneurosis with a nontoxic adenoma of the thyroid gland; another had psychoneurosis and adequately treated latent asymptomatic syphilis with no physical signs pointing to this condition. Three patients had arthritis (not rheumatic fever); one had a postinfluenzal asthenia; another had a transient intestinal obstruction (the cardiac study was made during his convalescence); and one patient had a fracture of his leg. There were, in addition, single cases of peptic ulcer, bronchitis, tracheitis, convalescent meningitis, hernia, extrasystoles, and nasal polyps. Seven patients failed to show evidence of any disease whatever.

Leads Involved.—In the patients showing the abnormalities of the T waves in the limb leads, the involvement in the majority (twelve) was in the sec-

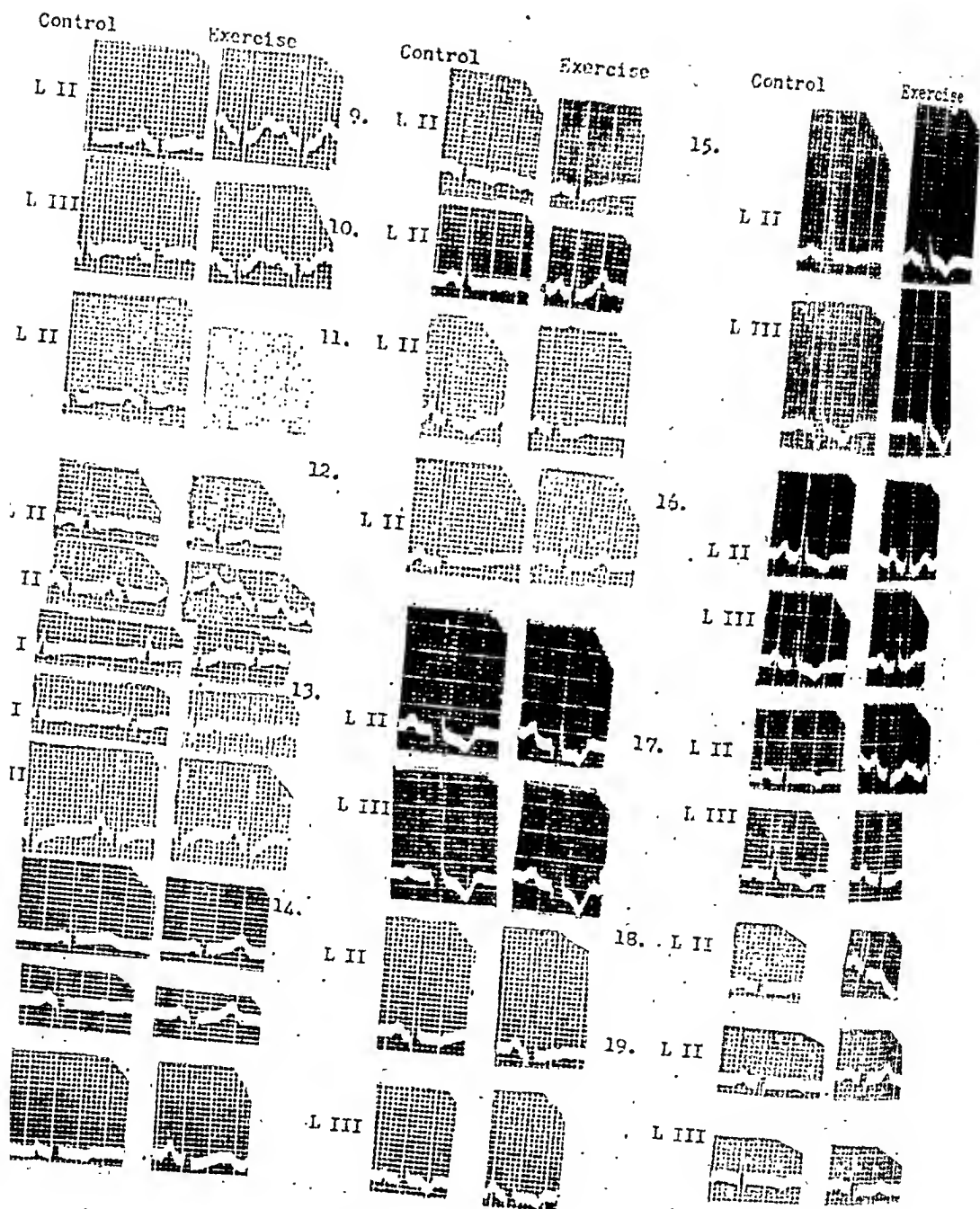


FIG. 1.—Limb leads.

ond lead. In eleven, Leads II and III showed the T-wave changes, three occurred in Leads I and II, one in Lead I alone, and one individual had abnormal T waves in the three limb leads.

There were twenty-three patients whose T_4 was abnormal. Of this group four also showed similar deviations in the T wave in the first lead. In one it occurred in combination with abnormal T_2 and T_3 . In one patient the T waves in all four leads were involved.

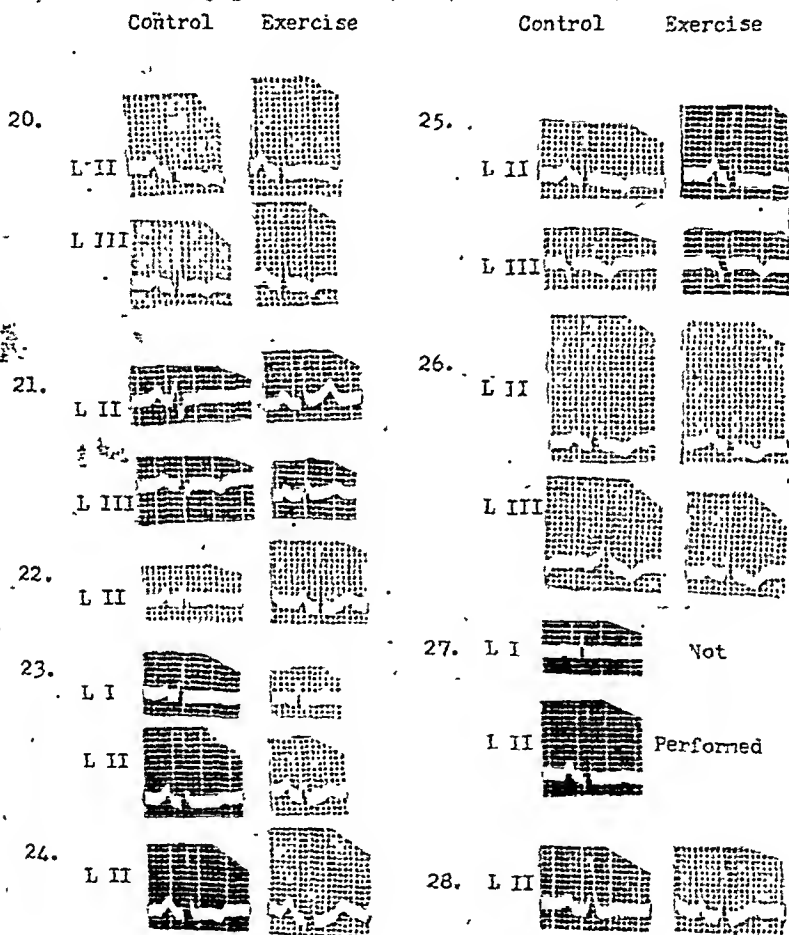
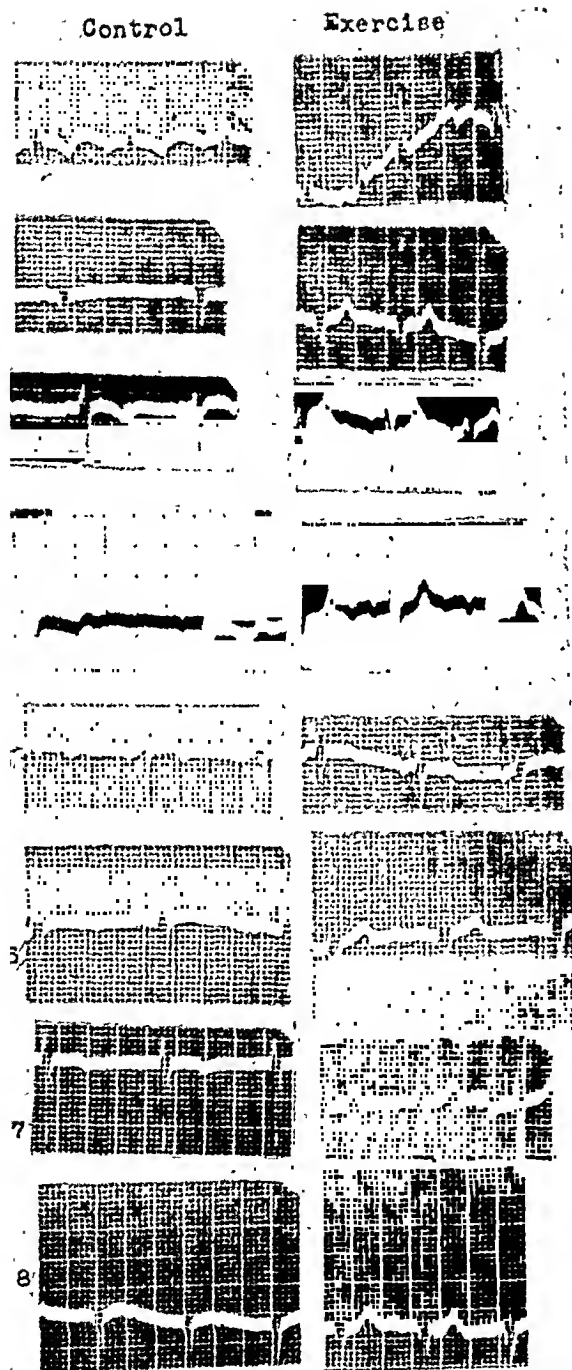


Fig. 2.—Limb leads.

Response to Exercise.—All but one of the patients were subjected to a standard amount of exercise (hopping on each leg fifty times) immediately following which an electrocardiogram was taken, again in the recumbent position. In those with the limb lead changes twenty-one revealed a reversal of the T wave to the upright position (Figs. 1 and 2). Six of the patients showed no change following this procedure.

Fig. 3.—Lead IV (CR₁).Fig. 4.—Lead IV (CR₄).

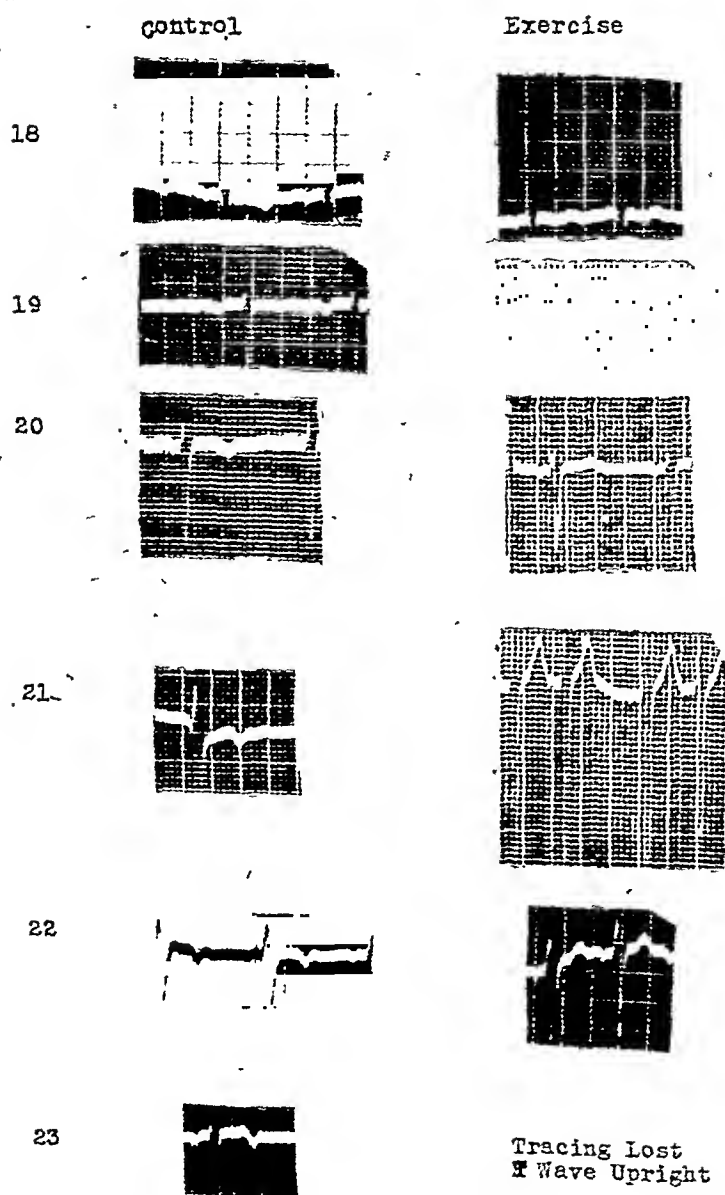


Fig. 5.—Lead IV (CR_4).

In those with the T_1 deviations the effects were even more striking; twenty-two of the twenty-three revealed a return to a normal T wave (Figs 3 to 5). In the entire group of fifty patients forty-three, or 86 per cent, showed a reversal of an abnormal to a normal T wave following physical exercise.

Heart Rates.—The ventricular rates in the limb lead group ranged between 50 and 138 per minute. Sixteen patients were below 90 per minute while twelve showed higher figures.

Among the T_1 group the largest number (six) had a ventricular rate between 70 and 79, four between 80 and 89, and four between 90 and 99. Seven patients had a rate between 50 and 69. Only six were above 90, and of these only two exceeded a rate of 100 beats per minute.

In summary, thirty-three of our patients, or 65 per cent of the group, had a ventricular rate under 90 per minute. This would tend to eliminate tachycardia as a factor in the production of the T-wave changes.

DISCUSSION

Current criteria for the normal electrocardiogram were published by Lewis and Gilder,² in 1912, and were based on a study of fifty-three young men. This field was left unexplored for many years, and no extensive investigation of the normal was undertaken until 1926 when Ferguson and O'Connell³ reported a series of electrocardiograms in 1,812 midshipmen at Annapolis without any evidence of heart disease (with three exceptions). There were eight instances where T_2 was inverted, diphasic, or isoelectric. Some of these were normal when a second tracing was taken. The mobilization of our Armed Forces during the war period gave the medical profession the golden opportunity of examining millions of young men, among which there were many thousands upon whom routine electrocardiographic studies were made. In this manner it became evident that bizarre cardiographic patterns usually indicative of grave myocardial disease were too frequently observed in young individuals devoid of any organic stigmas.

Hall, Steward, and Manning⁴ studied a group of 2,000 "normal" aviators and found nine with electrocardiograms of an abnormal pattern. Included among these were negative T_1 or T_2 or both and bundle branch block. Graybiel and associates⁵ reviewed the findings in 1,000 young, healthy aviators and discovered two with inverted T waves in the second lead but none in the first or fourth leads. T_2 in one was biphasic.

Our results in fifty-one patients constitute an addition to the list of electrocardiographic abnormalities in "normal" young people (twenty-eight involving limb leads, twenty-three with chest lead changes).

It is evident therefore that abnormalities of the T wave in "normal" individuals occur with fair incidence. Not so clear, however, is the mode of production of this aberration in the electrocardiogram and the significance, if any, to be attached to this phenomenon. It may be argued that the unusual T wave is the first indication of heart disease in these people, and disabling

signs or symptoms are sure to appear in the future. This opinion cannot be refuted in toto until these patients have been followed over a number of years; however, such a grave prognosis seems very unlikely.

Yet, if we are to base these changes on functional grounds, what are the factors involved? It is well known that the height of T waves is lowered or that the T waves can become inverted when the subject's position is changed from the recumbent to the sitting or standing attitude.⁶⁻⁸ Our patients were all recumbent when their tracings were taken. The location of the diaphragm is presumed to be a factor involved in these aberrations. High diaphragms can be expected in the sthenic type of patient and a lower position in those of the slender habitus. In our series this would not be applicable, as we encountered all types of body build, the largest group (twenty-six) being made up of those of medium habitus.

Imbalance of the autonomic nervous system has been suggested as an element in the production of unusual T waves. Wendkos⁹ studied a group of soldiers and concluded that the T-wave changes could be attributed to predominance of either vagal or sympathetic tone. Graybiel and White¹⁰ found inverted T waves in Leads II in seven instances of neurocirculatory asthenia.

By far the largest number of our series (60 per cent) fall in the "nervous" group who demonstrated unequivocal signs of derangement of the autonomic nervous system. To attribute the faulty T waves in these individuals to sympathetic or parasympathetic imbalance seems rational. The attempt to affect this system artificially through the medium of standardized exercise brought a restitution of the normal T waves in 86 per cent of our patients. This finding lends support to the suggestion that we were dealing basically with a functional situation.

We do not feel that emotional factors at the time the electrocardiographic examination was undertaken played any part in our findings. No outward apprehension was noted during this period and, as has been noted, tachycardia was not a dominant feature in our patients. Thirty-three of our group had ventricular rates of 90 per minute or less while eighteen were above that figure. A "fear reaction"¹¹ was not observed at any time and played no part in the picture presented here.

When one considers that seven of our patients showed no evidence of any disease process whatever and that thirteen others were suffering from ailments (rheumatic arthritis, transient intestinal obstruction, a fracture) unrelated to the heart, it is conceivable that we are merely dealing with a physiologic variant and that our criteria of the "normal" must undergo reconsideration. It is plausible that the wide range of the normal electrocardiogram has not been adequately explored. This would seem reasonable when we remember that the standards originally established by Lewis and Gilder² were based on the study of only fifty-three individuals. This statement is supported by the fact that in six of our patients (Patients 13, 17, 18, 21, 22, 27, Table I) tracings taken anywhere from two hours to ten days later showed a spontaneous reversal of the T wave to normal (Fig. 6). Similar findings were recorded

by Ferguson and O'Connell.⁵ Dupuy¹² advances such a thought in a presentation of five cases with T-wave abnormalities in other leads than in the third.

The value of exercise or sympathetic stimulation in the differential diagnosis of organic or functional heart disease remains a matter for further study. Without a control group of patients known to have organic heart disease, no conclusions are warranted.

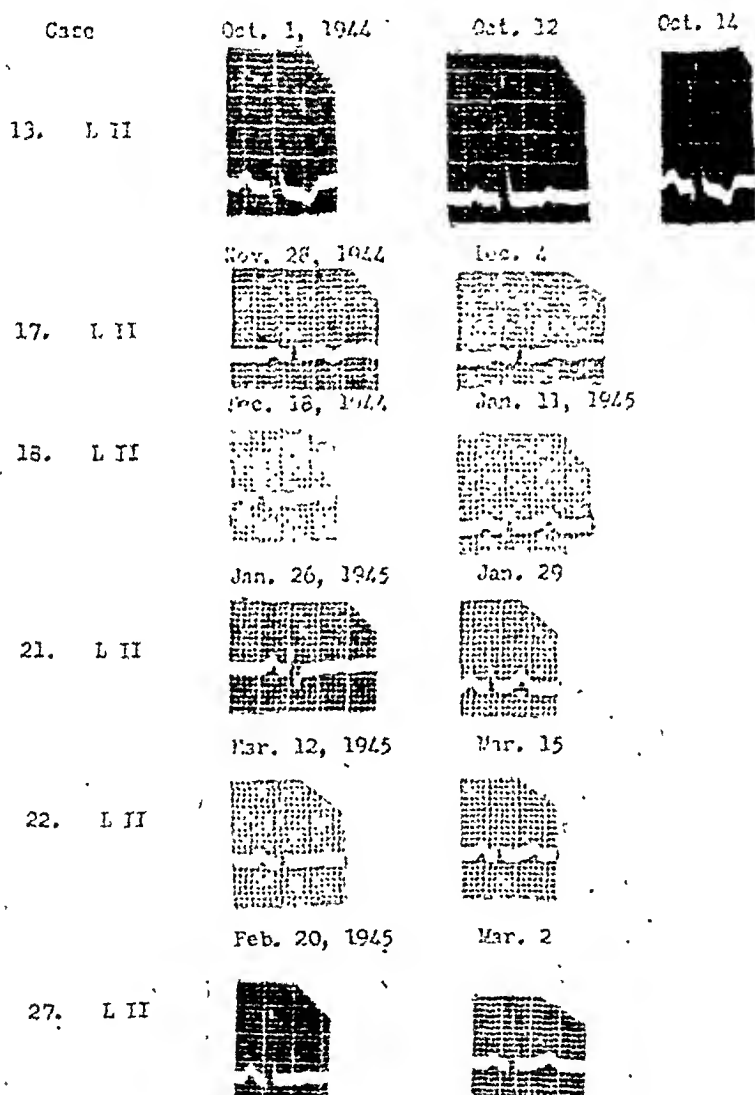


Fig. 6.—Spontaneous reversals of T waves.

The case history of one patient (Patient 25, Table I) is described in more detail because of several unusual features. This man was 29 years of age and entered the hospital because of frequent bouts of sharp pains in his chest for the previous two years. These pains were noted while at complete rest and

at other times during exercise; they lasted for about twenty minutes and were occasionally accompanied by "hard breathing." In the patient's words, he was unable "to get in enough air." Otherwise, he was in good health, except that he had always been of a "nervous disposition" and quick-tempered. Nothing in his past history was suggestive of heart disease nor of any rheumatic manifestation.

There were no abnormal signs elicited on physical examination. Fluoroscopy of the chest indicated no enlargement of any of the cardiac chambers. The Kahn test was positive, and he received an intensive course of penicillin

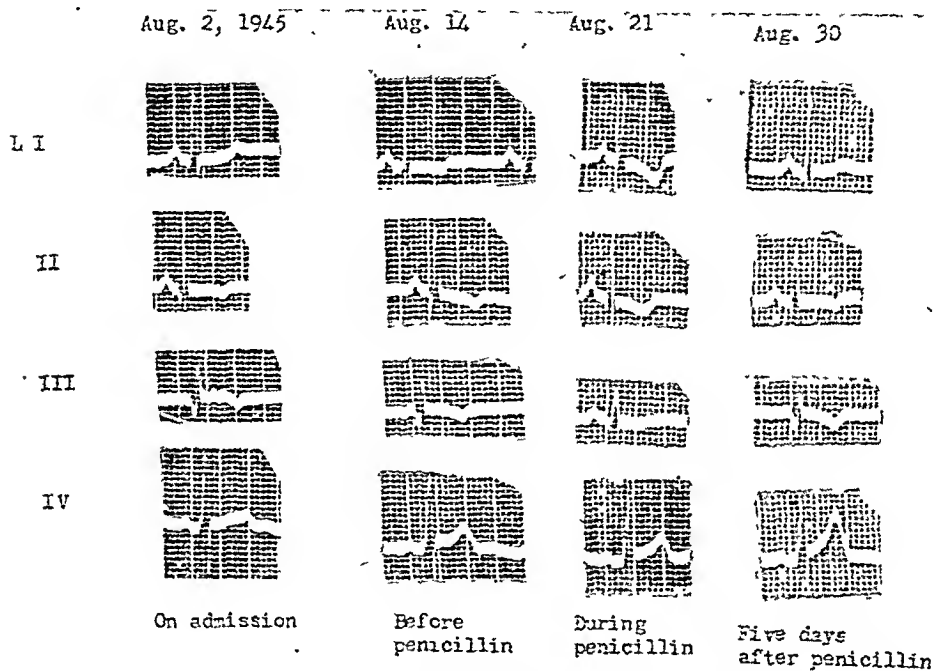


Fig. 7 (Patient 25).—Note changes in T_1 from upright to inversion and return to normal within a month. Changes during penicillin therapy considered coincidental.

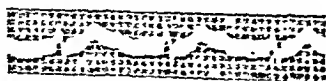


Fig. 8 (Patient 17).—P-R interval, 0.28 second.

therapy (2,400,000 units in a seven-day period). The sedimentation rate was elevated (33 mm. in an hour) on admission and was 22 mm. two months later. During the last month of his hospitalization it was normal (10 mm. or less).

The outstanding feature in the electrocardiograms was the inversion of T_2 and T_3 (Fig. 7). This persisted throughout his course and was unaffected by exercise or the administration of ergotrate, 0.4 mg. intravenously, or full doses of atropine. An electrocardiogram taken during the period of penicillin treatment revealed an inversion of T_1 (Fig. 7). This disappeared after a

few weeks. The significance of this finding and the possible relationship of the electrocardiographic pattern, as well as the elevated sedimentation rate to his syphilis and the penicillin employed in its treatment, are difficult to evaluate. It is extremely questionable that the penicillin played any part in the production of the inverted T_1 .

It must be remembered that during the entire period the patient was ambulatory, although the symptomatology was the same and the examination was not productive of abnormal findings. The course in the hospital was otherwise uneventful, and on discharge after three months the symptoms continued unabated. It was finally concluded that the subject did not have organic heart disease but that his symptoms were functional in origin.

SUMMARY AND CONCLUSIONS

1. Five thousand, five hundred and twenty electrocardiographic studies made on 4,810 patients at a large Army regional hospital revealed fifty-one, or 1.0 per cent, of this total with abnormal T waves despite the absence of other criteria of heart disease. In twenty-three the bizarre T waves were confined to the chest leads and in twenty-eight appeared in the limb leads.

2. The subjects studied were of both sexes ranging in age from 19 to 47 years, the average being 29.9 years.

3. Constitutional types varied widely between the sthenic and asthenic; the majority (twenty-six, or 65 per cent) were of medium build.

4. A majority of the subjects in the series (thirty-one, or 6 per cent) presented definite psychoneurotic tendencies. However, thirteen had ailments unrelated to heart disease, and in seven no evidence of illness of any kind was determined.

5. In forty-three out of fifty patients (86 per cent of the total) standard exercise was successful in converting the bizarre T waves to normal.

6. The underlying theoretical considerations for the abnormal T waves were discussed. The possibility that at least in some instances these were merely physiologic variants was ventured. Many were undoubtedly based on autonomic nervous system imbalance.

7. One should be aware of a "normal" inversion of the T wave in order to avoid a mistaken diagnosis of heart disease.

I wish to express my appreciation for the technical assistance rendered by Lieutenant Gladys Eklund, Army Nurse Corps, and Corporal Ted Weible.

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OBSERVATIONS ON THE EFFECT OF THEOPHYLLINE AMINOISOBUTANOL IN EXPERIMENTAL HEART FAILURE

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XANTHINE drugs, particularly theophylline preparations, have had wide clinical use in cardiac disease because of their diuretic effect, their beneficial action in cardiac dyspnea, and their coronary dilator properties. The xanthine drugs also stimulate the myocardium directly,^{1,2} although this action usually has not been stressed as of clinical importance.^{4,5}

In a previous report Steinberg and Jensen⁶ described their study of the effect of theophylline aminoisobutanol in patients with congestive heart failure. They found that administration of the drug in such patients usually lowered the venous pressure and resulted in shortening of the circulation time. The diminution of venous pressure was striking when it was initially elevated, but it was nevertheless often further diminished even when normal. The observations suggested that these beneficial actions result from: (1) direct myocardial stimulation by the drug; (2) improved myocardial function resulting from increased coronary flow; (3) action of the drug to produce peripheral vasodilatation; or (4) a combination of these actions. The results of Steinberg and Jensen were so striking that it was decided to investigate certain pharmacologic actions of theophylline aminoisobutanol in experimental heart failure, with the hope of clarifying the mechanism of the clinical effects noted by them.

METHOD

In these experiments, where heart failure was to be produced and then studied with respect to drug effect, it was desirable to record all features of myocardial performance as accurately as possible. The heart-lung preparation was chosen as the best means of observing these factors. In Fig. 1 is illustrated the experimental setup in principle.

Heart-lung preparations were made in the usual way and then removed from the body of the dog. Tensions within the right and left auricles were measured by simple manometers connected to the superior vena cava and through a cannula inserted into one of the pulmonary veins, respectively. Cardiac output was continuously measured by a siphon recorder. A rough estimate of cardiac size was obtained by means of a graduated pair of outside calipers stitched to the epicardium. Inflow of blood into the left circumflex coronary artery was recorded by the differential pressure orifice meter devised by Gregg

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and Green,⁷ the light beam from which was projected into an electrocardiographic photokymograph. Standardization of the instrument and quantitative interpretation of inflow curves have been described⁷ and were carefully followed in these experiments. Arterial blood pressure was obtained using a Wiggers optical manometer; the light beam from this manometer also was projected into the kymograph and was recorded simultaneously with the coronary inflow curve on a bromide film.

When control observations had been made, heart failure was produced by the careful administration of 20 per cent chloral hydrate (1 c.c. every three minutes) to the perfusing blood. Approximately 0.8 Gm. of chloral hydrate was usually sufficient to produce definite heart failure without causing irretrievable cardiac damage.⁸ With heart failure thus established, theophylline

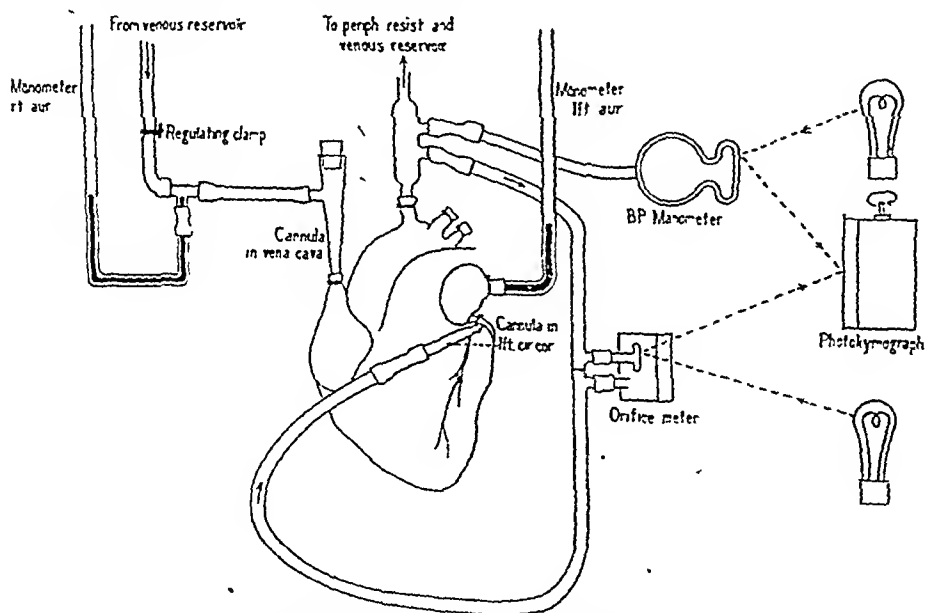


Fig. 1.—Diagrammatic sketch of heart-lung preparation and the instruments applied to it for the evaluation of myocardial function. Simple manometers are connected into the tube from the venous reservoir and into the left auricle. The flowmeter records inflow into the left circumflex coronary artery. Calipers used to estimate cardiac size and siphon for constant recording of cardiac output are not shown.

aminoisobutanol* (0.06 Gm.) was injected into the perfusing blood. The effects of the drug were observed until cardiac function became "stabilized." These experiments were performed on fourteen heart-lung preparations with consistent findings throughout.

As the study developed, it became desirable to ascertain changes of blood flow through edematous lungs in the course of heart failure and to note the effect of theophylline aminoisobutanol on pulmonary edema and congestion. In these observations the modified heart-lung preparation of Dusser de Barenne⁹ was employed. In such a preparation blood is carried from the venous reservoir

*Wm. S. Merrell Co.

directly to the pulmonary artery. The left ventricle performs its normal complement of work; the right ventricle performs no work but serves as a receptacle for almost the whole of the coronary venous blood (see Fig. 2). At any time the arterial blood may be shunted around the artificial peripheral resistance and collected, together with coronary venous blood, in a recording siphon. These combined flows, therefore, represent total pulmonary flow. Heart failure was produced by using 20 per cent chloral hydrate as in the foregoing experiments, except that sufficiently more chloral hydrate was given to bring the heart to a standstill. Intense pulmonary edema and congestion occurred. The rate of blood flow through the lungs was then determined. Theophylline aminoisobutanol (0.06 Gm.) was added to the perfusate as the rate of blood flow through the lungs was continuously observed. Such observations were carried out on four de Barenne preparations.

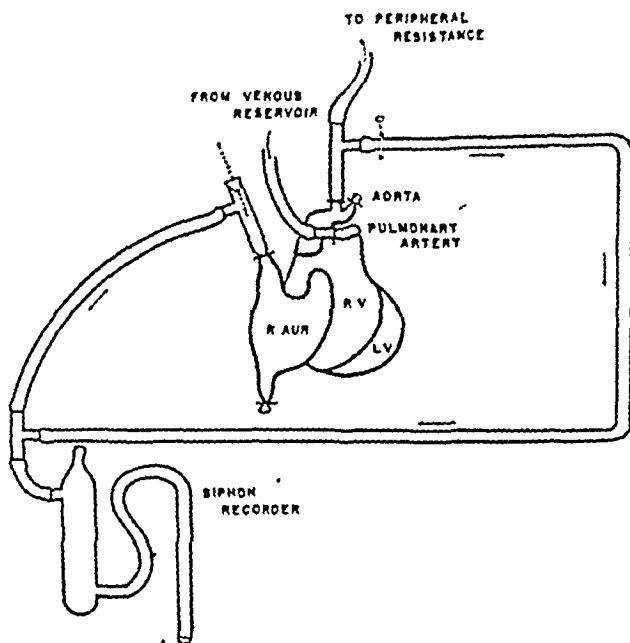


Fig. 2.—Schematic diagram of the modified heart-lung preparation of Dusser de Barenne (lungs are not shown). Blood from the venous reservoir is carried directly to the pulmonary artery, passes through the lungs, and is pumped through a peripheral resistance by the left ventricle. The right ventricle does no work. A cannula in the superior vena cava collects a large part of the coronary venous blood. The stream to the peripheral resistance may be clamped at will, and blood from the left ventricle may be shunted to the siphon-recorder and collected, together with coronary venous blood. The combined flows represent total pulmonary flow (cf. text).

RESULTS

Production of Heart Failure in the Heart-Lung Preparation.—When chloral hydrate had been added to the blood in the heart-lung, failure of the myocardium was manifested in the following series of events (see Table I): (1) Dilatation of the heart occurred, as shown by an increase in the diameter of the ventricles. Simultaneously, a rise of pressure within the left auricle was observed over a period of from fifteen to thirty minutes to approximately 10 to 11 cm. of water.

TABLE I. REPRESENTATIVE PROTOCOL OF EXPERIMENTAL HEART FAILURE IN HEART-LUNG PREPARATION AND THE EFFECT OF THEOPHYLLINE AMINOISOBUTANOL

EVENT	CARDIAC OUTPUT (C.C. PER MIN.)	BLOOD PRES- SURE	RIGHT AURICLE (CM. OF H ₂ O)	LEFT AURICLE (CM. OF H ₂ O)	VEN- TRICU- LAR DI- AMETER (CM.)	RATE	CORO- NARY INFLOW CYCLE (C.C.)	CORO- NARY INFLOW (C.C. PER MIN.)	REMARKS
Control	630	95/60	4.4	4.9	5.5	166	0.23	38.1	
Chloral hy- drate (0.8 Gm. 12 m)	—	—	—	—	—	—	—	—	
Heart fail- ing	480	90/60	6.6	11.0	6.7	111	0.35	38.8	Pulmonary edema and congestion
Theophyl- line (3 min.)	600	100/60	5.5	6.6	5.5	115	0.32	36.8	Myocardial stimulation; pulmonary edema sub- sides

Usually, right intra-auricular tension was not materially altered unless terminal severe myocardial failure supervened. Heart failure produced in this way was manifested, therefore, predominantly as left ventricular failure. (2) With dilatation of the heart a fall in cardiac output (from 25 to 40 per cent) occurred. The blood pressure diminished. The heart rate decreased. (3) Coronary arterial inflow *increased* during the course of cardiac failure exemplified by increase in inflow during each cardiac cycle. The decrease in heart rate usually rendered the net inflow per minute essentially unchanged. (4) The lungs showed intense congestion and edema with emphysema, parenchymal hemorrhage, and fluid in the tracheal cannula. Heart failure was not permitted to become more severe, as irremediable failure than occurred with loss of the experiment.

Effect of Theophylline Aminoisobutanol Upon Heart Failure.—The course of events following the administration of theophylline aminoisobutanol may be followed in Table I. Without exception the first measurable effect of the drug (occurring within one minute) was the rapid clearing of pulmonary edema and congestion. The run-off from the lungs appeared to increase so quickly that, momentarily, the left intra-auricular tension was driven up an additional 6 to 10 cm. of water, and transient further dilatation of the heart occurred. Thereafter, an obvious marked stimulation of myocardial action was observed. The heart beat vigorously. Within a few beats dilatation of the heart was completely removed. Cardiac output increased. Within three minutes the heart had resumed its "normal" state, and the entire preparation appeared to function as effectively as in the control period. Coronary arterial inflow was not essentially changed during the period of recovery from heart failure. Inflow during each cardiac cycle tended to decrease, and net inflow remained essentially unaltered with the rise in heart rate.

Effect of Theophylline Aminoisobutanol on Pulmonary Edema and Congestion in the de Barenne Preparation.—In the foregoing experiments the administration of theophylline appeared to cause a remarkably rapid clearing of pulmonary edema. In this series of experiments pulmonary blood flow was greatly

TABLE II. RECORD OF TWO EXPERIMENTS USING DE BARENNE PREPARATION*

PULMONARY FLOW—CONTROL (C.C. PER MIN.)	LUNG CONGESTED—HEART STOPPED (C.C. PER MIN.)	AFTER THEOPHYLLINE NO HEART BEAT (C.C. PER MIN.)
321	191	256
400	300	360

*Blood flow through the lungs before and during pulmonary congestion and edema produced by myocardial failure. The effect of theophylline aminoisobutanol on blood flow through the lungs when congested and edematous.

reduced during the severe pulmonary congestion and edema resulting from myocardial failure and standstill (see Table II). With the addition of theophylline (0.06 Gm.), there was an immediate increase in blood flow through the lungs with prompt subsidence of congestion and edema. By this means pulmonary flow was restored to approximate control values.

DISCUSSION

From the experimental data at hand, the most striking—and probably the most important—effect of theophylline aminoisobutanol in heart failure is its action upon the myocardium. In all of the experiments the drug quickly induced marked, obvious vigor of ventricular contraction and prompt removal of cardiac dilatation. This phenomenon had been noted by other investigators¹⁻³ upon normally beating experimental hearts, and it was attributed by Heathcote¹ to its action directly upon the heart muscle. The question was then raised as to whether improvement in myocardial function resulted from coronary vasodilatation produced by the drug, with a corresponding increase in myocardial blood supply. That the xanthine drugs provoke coronary dilatation has been amply shown.¹⁰⁻¹² However, in these experiments an increase of coronary inflow (in each cardiac cycle) occurred in the course of failing integrity of the myocardium, despite a falling cardiac output and blood pressure and a rising tension in the left auricle. Since net coronary arterial inflow appears to be dependent in great part upon cardiac output¹³ in normally beating experimental hearts, the increased inflow noted here is possibly due to peripheral coronary dilatation. These findings are in agreement with the experience of others. Gregg and Green¹⁴ observed marked increase in coronary flow (under constant perfusion pressure) following transient ischemia of the heart muscle; more recently Green and Wegria¹⁵ demonstrated an increase in myocardial irrigation during anoxia of the myocardium. It seems probable, therefore, that the effect of theophylline upon the coronary vascular bed in heart failure, which is already dilated, is largely annulled and that the drug exerts its beneficial action by direct stimulation of myocardial contractions. Visseher¹⁶ has pointed out that in myocardial decompensation rising pressures in the right auricle and ventricle may impede the escape of blood from the coronary venous system to curtail coronary flow generally. In our preparations there was no significant rise of right intra-auricular tension. Therefore, it cannot be stated from our data whether impedance of coronary flow may have occurred had the circulation been intact, or what the effect of theophylline may be under such circumstances. The latter consideration is a recognized disadvantage of the heart-lung preparation.

In the light of available experimental evidence the mechanism of the removal of pulmonary edema by theophylline is difficult to evaluate. Friedberg, Katz and Steinitz¹⁷ found in trained, unanesthetized dogs that the administration of aminophylline led to a mild, sustained elevation of mean pulmonary arterial tension. In their opinion the beneficial action of theophylline clinically could not be explained by the relief of congestion. However, they further suggested that theophylline might have an opposite effect when pulmonary congestion is present. On the other hand, Bock¹⁸ observed that the xanthines increased blood flow through isolated lungs. Our observations suggest that marked vasodilatation occurs when theophylline is administered in pulmonary congestion. Blood impounded in the lungs escapes, and fluid rapidly leaves the alveolar air spaces to re-enter the circulation. Whether other factors, such as capillary permeability, are altered by the drug directly cannot be stated at present. In the heart-lung preparation the additional factors of improved myocardial function and release of increased pulmonary venous tension are also undoubtedly important in the removal of pulmonary edema.

Experimental evidence further indicates that theophylline preparations produce peripheral vasodilatation. Many years ago Phillips and Bradford¹⁹ and Beco and Plumier²⁰ demonstrated increased blood flow through the renal vessels and other parts of the vascular bed by xanthine drugs. Sollman and Pilcher²¹ and Phillips and Bradford¹⁹ showed, by oncometric methods, that these drugs increased the size of the spleen in experimental animals. From the clinical standpoint, therefore, it seems probable that in addition to enhancing myocardial function and improving pulmonary blood flow theophylline also dilates, to some extent, the peripheral vascular bed. These factors, taken together, may improve the failing heart and circulation.

CONCLUSIONS

1. Heart failure was produced in the heart-lung preparation using chloral hydrate as a myocardial intoxicant. The administration of theophylline aminoisobutanol provoked striking stimulation of myocardial contraction with prompt restoration of effective function of the heart muscle.

2. Pulmonary edema and congestion, in the course of failure in the heart-lung preparation, appeared to be removed promptly by theophylline aminoisobutanol. This phenomenon was further examined in the modified de Barenne heart-lung preparation. It was found that pulmonary blood flow was greatly curtailed in pulmonary congestion from heart failure. The addition of theophylline aminoisobutanol caused a rapid removal of pulmonary edema and congestion with restoration of free blood flow through the lungs.

3. We find no evidence from these observations that improvement in the failing experimental heart is dependent upon dilatation of the coronary arterial system by theophylline.

4. It is suggested that clinical benefit with theophylline aminoisobutanol in patients with heart failure results from improved myocardial function and pulmonary flow. Peripheral vasodilatation induced by the drug may also further benefit the disturbed circulation in such patients.

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THE EFFECT OF THEOPHYLLINE AMINOISOBUTANOL ON THE CIRCULATION IN CONGESTIVE HEART FAILURE

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INTRODUCTION

IT IS generally accepted that theophylline has important but still incompletely known effects on the cardiovascular system.^{1,2} This paper is concerned with some of these effects in the presence of congestive heart failure. We were first impressed with the decrease in venous pressure which we observed following the administration of theophylline aminoisobutanol, especially when the venous pressure was increased. This effect has been reported in the literature but has not been thoroughly investigated. Greene, Paul, and Feller³ had noted it when studying the effect of theophylline upon the mechanism of dyspnea. Robertson and Faust⁴ recommended the use of theophylline for lowering the venous pressure in congestive heart failure, but they employed doses too small to obtain significant effects. We could find no evidence that the subject had been further investigated. We also noted that the arm-to-tongue circulation time was shortened after administration of this drug, a finding not previously reported in the literature. A clinical study was therefore undertaken in order to investigate further these changes in venous pressure and circulation rate which follow the administration of theophylline.

METHOD

Theophylline aminoisobutanol was given intravenously in doses of 0.24 and 0.48 Gm. For comparison, theophylline with ethylenediamine (aminophylline) was used in a smaller number of cases. A few tests were made with the sublingual administration of 0.6 mg. of glyceryl trinitrate (nitroglycerin).

The venous pressures were determined by a modification of the method of Moritz and Tabora.⁵ The apparatus used is shown in Fig. 1. The precautions recommended by Lyons, Kennedy, and Burwell⁶ were closely observed. The needle was left in the vein during the entire period of observation. Clotting was prevented by adding a 2.5 per cent solution of sodium citrate at the rate of from 6 to 8 drops per minute. Theophylline and the drugs used for the determination of the circulation time were injected through a three-way stopcock. Therefore with one venepuncture it was possible to follow the venous pressure for one or two hours, introduce the drugs to be tested, and determine the circulation time as often as desired. The spontaneous changes of the venous pressure were studied by this method for varying periods of time, generally about twenty minutes, before the introduction of any drug. After a drug had

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been administered readings were taken every few minutes until the effect had worn off or the test had to be interrupted for other reasons, especially restlessness of the patient.

The arm-to-tongue circulation time was determined before and one or more times after the drug had been administered. For most tests decholin was used. When this was not available, from 2 to 3 c.c. of a 25 per cent solution of magnesium sulfate or 2 c.c. of a saturated solution of saccharin were used instead. Saccharin was found less suitable as it may cause a painful spasm of the vein into which it is injected. This gives rise to a disturbing temporary elevation of the venous pressure.

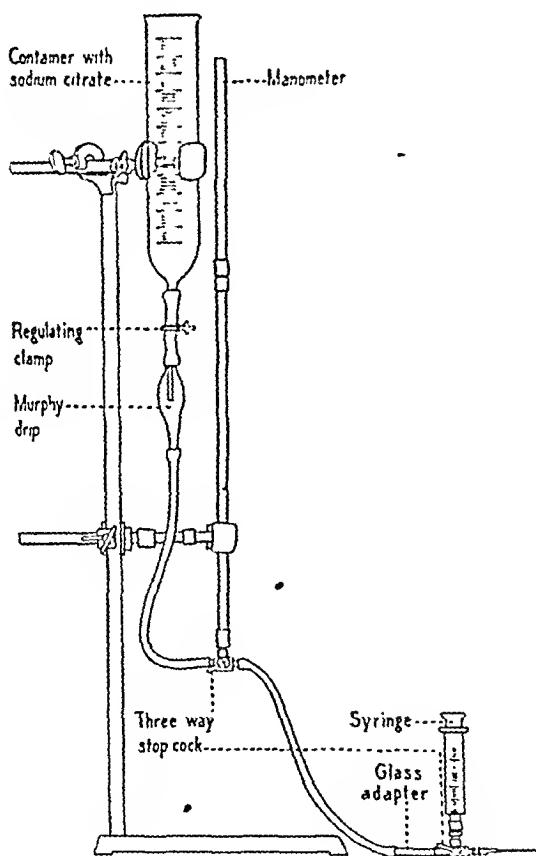


FIG. 1.

Plasma volume determinations* were carried out according to the method of Gibson and Evans⁷ as modified for the photoelectric colorimeter by Gibson and Evelyn.⁸ Gibson and Evans found that after the injection of from 15 to 20 mg. of Evans blue the dye was completely mixed with the blood in from ten to twenty minutes. However, in our cases of congestive heart failure the

*We are greatly indebted to Dr. R. Dubach, for her assistance in carrying out these determinations.

circulation was retarded and from forty-five to sixty minutes were allowed to elapse. After this period three blood specimens were drawn at from seven- to ten-minute intervals. Then 0.48 Gm. of theophylline aminoisobutanol was injected, and from five to ten minutes thereafter three more blood specimens were drawn. The dye concentrations were determined by means of the photoelectric colorimeter, and the plasma volume was computed following directions by Gibson and Evelyn. Venous pressure determinations were made simultaneously.

RESULTS

Preliminary Observations.—In fourteen patients with congestive heart failure, heart rate, arterial blood pressure, venous pressure, arm-to-tongue circulation time, and vital capacity were determined before and fifteen minutes after the intravenous administration of 0.24 or 0.48 Gm. of theophylline aminoisobutanol. In almost all cases the administration of the drug was followed by a decrease of venous pressure and shortening of the circulation time. Consequently it was decided to investigate further these two effects. Heart rate, arterial pressure, and vital capacity remained essentially unchanged.

Venous Pressure

Changes of Venous Pressure Apart From Drug Effect.—It soon became apparent that the venous pressure varied widely apart from any drug effect. Frequently the highest reading was obtained immediately after the needle had been introduced into the vein. Within a few minutes the pressure fell to a lower level where it remained stationary. Pogany⁹ states that the mechanical irritation of venepuncture may give rise to a spastic contraction of the vein with a transient elevation of venous pressure. This phenomenon was not observed in all patients, but it could usually be repeated in the same individual. Relaxation or sleep caused a spontaneous fall in venous pressure. Coughing, vomiting, or other acts associated with an elevation of the intrathoracic pressure

TABLE I

FALL IN VENOUS PRESSURE (MM. OF WATER)	CONTROL GROUP (NO. OF CASES)	THEOPHYLLINE AMINOISOBUTANOL GROUP (NO. OF CASES)	AMINOPHYLLINE GROUP (NO. OF CASES)	NITROGLYCERIN GROUP (NO. OF CASES)
1 to 19	35	7		1
20 to 39	24	16	4	5
40 to 59	3	9	4	
60 to 79	3	6	8	4
80 to 99	0	2	2	2
100 to 119	0	1	2	1
120 to 139	0	0	1	0
140 to 159	2	0		0
Total number	67	39	0	0
Range	2 to 128	8 to 77	21	13
Mean	23.6	36.7	11 to 118	18 to 88
Standard deviation	22.2	16.5	48.0	47.8
Median	18.0	33.0	27.8	20.2
Difference of means:			48.0	39.0
Standard error		3.47	3.67	3.91

In the control group the fall of venous pressure was measured from the highest to the lowest reading obtained. When drugs were given it was measured from the reading just before the drug was administered to the lowest reading obtained within thirty minutes.

led to a steep rise of the venous pressure, followed by a rapid decline to the original level. The venous pressure usually rose during the apneic phase of Cheyne-Stokes respiration as previously reported by Meyer and Middleton.¹⁰ Sometimes the pressure varied without apparent cause.

The following observations were made in sixty-seven control periods lasting from six to fifty-seven minutes, mostly about twenty minutes. The maximum variation from the highest to the lowest reading ranged from 2 to 128 mm. of water, with an average of 23.6 mm. of water (Table I).

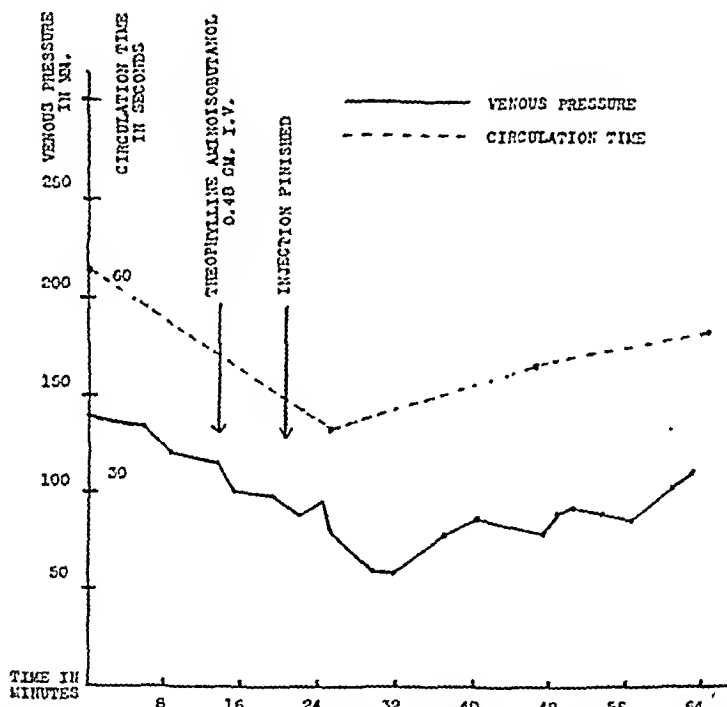


Fig. 2.

Theophylline Aminoisobutanol.—In the majority of cases the injection of theophylline aminoisobutanol caused an abrupt fall of the venous pressure. The drop usually began during the course of the injection which, for reasons of safety, was made slowly. Often the lowest point was reached with the end of the injection. In other cases the pressure continued to fall; it reached its low almost always within the first one-half hour. Thereafter the pressure fluctuated for some time in the vicinity of the lowest reading or it began to rise again. (Fig. 2.) Often changes attributed to the drug were obscured by changes due to other factors which sometimes caused rises before they could otherwise be expected.

To determine the extent of the decrease, the average of the last two readings before the drug was given was compared with the lowest reading obtained within thirty minutes. In thirty-nine cases in which 0.48 Gm. of theophyl-

line aminoisobutanol was given, the venous pressure decreased from 8 to 77 mm. of water with an average decrease of 36.7 mm. This is considerably more than the average maximum spontaneous variations of 23.6 mm. of water.

The distribution of the spontaneous decreases in venous pressure was compared with the distribution of decreases which followed the injection of theophylline aminoisobutanol and also with those which followed aminophylline and nitroglycerin (see below). The statistical analysis of these data is recorded in Table I. The difference between the means of the control group and the theophylline aminoisobutanol group is 3.47 times the standard error. This indicates that the differences are significant. This is notwithstanding the fact that the differences in the control series were the maximum ones obtainable, while the drug effect was measured consistently as the difference between the reading preceding the drug and the lowest reading obtained.*

The fall of the venous pressure was generally the more extensive the higher the pressure before injection. However, no quantitative correlation could be established between the height of the original venous pressure and the amount of decrease, expressed either in absolute figures or in percentages. This is not surprising since we were faced with a reaction which is subject to so many outside influences.

TABLE II

DRUG	REACTION END- ING WITHIN SIXTY MINUTES	REACTION LAST- ING MORE THAN SIXTY MINUTES	TEST TERMINATED BEFORE SIXTY MINUTES AND BEFORE END OF REACTION	TOTAL
Theophylline aminoisobutanol	19	4	15	38
Aminophylline	7	7	7	21
Nitroglycerin	7	2	4	13

The duration of the effect was often difficult to determine because it might be obscured by increases in venous pressure due to some of the factors previously mentioned. Sometimes restlessness necessitated premature termination of the test.

Arbitrarily the end of the reaction was thought to occur when three consecutive readings were within 10 per cent of the last reading before the drug was given. For comparison with the effect of other drugs, it was thought practical to divide the cases into those in which the effect ended within sixty minutes, those in which it persisted beyond sixty minutes, and those in which outside factors caused termination of the test within sixty minutes, yet before the reaction ended (Table II).

Aminophylline.—In twenty-one cases 0.48 Gm. of aminophylline was injected intravenously. The reaction followed the same pattern as described for theophylline aminoisobutanol. The decline of the venous pressure from the pre-injection to the lowest reading ranged from 11 to 118 mm. of water with an aver-

*We are indebted to Dr. E. Gurney Clark, for his assistance in the statistical analysis of our data.

age of 48.0 mm. (Table I). The duration of the aminophylline effect on the venous pressure is seen in Table II. The impression that it lasts longer with this drug than with theophylline aminoisobutanol or nitroglycerin rests upon too few observations to be convincing.

Nitroglycerin.—In thirteen cases 0.6 mg. of nitroglycerin was given sublingually. Usually the pressure began to fall steeply within one minute after the administration of the drug. The decrease from the reading before nitroglycerin to the lowest value ranged from 18 to 88 mm. of water and averaged 47.8 mm. for the thirteen cases (Table I).

Failure of Response.—The cases in which the venous pressure failed to decrease fell into two groups. The larger group comprised cases in which the venous pressure was not elevated prior to drug administration. In these there was little opportunity for a significant decrease. The second group contained cases in which the venous pressure was definitely above normal but responded poorly to the drug, that is, fell to less than 20 mm. of water. We encountered five such cases in the theophylline aminoisobutanol group, two in the aminophylline, and none in the nitroglycerin group. The reason for the failure to react was uncertain, but most of the patients were markedly decompensated and failed to react to other therapeutic measures as well.

Repeated Tests.—In many cases the test was repeated once or several times in the same patient on different days in order to test the individual constancy of pattern. Again, the great spontaneous variations made comparison difficult. The venous pressure changed so much from day to day that rarely were the starting points the same.

Circulation Time

The arm-to-tongue circulation time was measured on 168 occasions. Being a "subjective" test it is influenced by the patient's ability to recognize the taste, but there was no doubt that theophylline aminoisobutanol often shortened the circulation time, more so the longer the original time. The second reading was taken approximately fifteen minutes after the beginning of the administration of the drug. The results are summarized in Table III.

TABLE III

DRUG	NUMBER OF CASES	DOSE	AVERAGE SHORTENING OF CIRCULATION TIME IN SECONDS
Theophylline aminoisobutanol	9	0.24 Gm.	10.4
Theophylline aminoisobutanol	20	0.48 Gm.	12.8
Aminophylline	9	0.48 Gm.	16.8
Nitroglycerin (sublingual)	9	0.6 mg.	8.6

There was relatively little difference between the effect of the various preparations. Perhaps nitroglycerin in the customary doses is somewhat less effective in accelerating the circulation. Not enough tests were made to determine how long the effect lasted, but we had the impression that it passed off in from thirty to ninety minutes or about simultaneously with the drop in venous pressure.

Comparison Between Circulation Time and Venous Pressure.—For the purpose of the following analysis the upper normal limits of the circulation time and venous pressure were considered to be twenty seconds and 120 mm., respectively. In our observations it was more common for the circulation time to be prolonged while the venous pressure was normal than for the reverse to occur. In only one case was the venous pressure elevated (158 mm.) while the circulation time was low (ten seconds); this patient had a marked anemia, a condition which accelerates the circulation. These observations would suggest that the slowing of the circulation time is a more fundamental change in congestive heart failure than is increase in venous pressure.

Following the drugs both venous pressure and circulation time were affected in most cases. Changes of less than five seconds in the circulation time or of less than 20 mm. for the venous pressure were not accepted as drug effects. In no case was the circulation time shortened without fall of the venous pressure; in eleven cases the venous pressure fell while the circulation rate remained unaffected. This observation again supports the impression that slowing of the circulation rate is more fundamental than increase of venous pressure in congestive heart failure. Four cases failed to respond in both respects.

Plasma Volume

Plasma volume determinations were carried out in six cases before and after the injection of 0.48 Gm. of theophylline aminoisobutanol. In no case

TABLE IV. DETERMINATION OF VENOUS PRESSURE AND PLASMA VOLUME BEFORE AND AFTER INJECTION OF THEOPHYLLINE AMINOISOBUTANOL
(PATIENT W. G.; AGE, 60 YEARS; DIAGNOSIS, ARTERIOSCLEROTIC HEART DISEASE)

TIME	VENOUS PRESSURE (MM. OF WATER)	PLASMA VOLUME (C.C.)
10:15	328	
10:21	288	
10:23	274	3,873
10:27	280	
10:29		
10:30	264	3,750
10:32	263	
10:37	272	
10:38	270	
10:39		
10:40	263	3,894
10:42	270	
10:42 to 10:46	Theophylline aminoiso- butanol, 0.48 Gm. intravenously	
10:46	232	
10:47	224	
10:48	220	
10:49		
10:51	226	3,894
10:52	222	
10:54	222	
10:56	226	
10:57		
10:59		
11:00	230	3,894
11:00	242	
11:02	254	
11:04	253	
11:05		

could a significant change in dye concentration and plasma volume be observed, even though the venous pressure fell as usually. This indicates that there was no important shift of fluid from the blood into the tissues during the period of observation. A representative protocol of one of these tests is reproduced in Table IV.

DISCUSSION

Prolongation of the circulation time and elevation of the venous pressure are fundamental factors in the pathogenesis of congestive heart failure. An improvement in the circulation will necessarily be associated with a relief of venous congestion and an acceleration of the circulation. Our observations establish that theophylline produces these effects and that this drug, therefore, should have a definite place in the treatment of congestive heart failure. The effect begins almost instantaneously but soon wears off. Therefore, the drug will be most useful in emergencies. It can be used to precede digitalis action, which is slower in producing an effect but which is more sustained.

On the basis of clinical experience the use of theophylline has been recommended in the acute phases of congestive heart failure. However, the literature contains only a few references to its effect on the venous pressure. The reports by Greene, Paul, and Feller³ and Robertson and Faust⁴ have been mentioned previously. Wilkins, Haynes, and Weiss,¹¹ have reported the fall of venous pressure which follows the administration of nitrites.

Our observations give no explanation of the mechanism by which theophylline produces the observed reduction of venous pressure and circulation time. Several possibilities have to be considered: (1) peripheral vasodilation, (2) strengthening of the myocardium, (3) indirect improvement of myocardial efficiency due to an increase of the coronary circulation, and (4) a combination of several or all of these factors.

The fact that the xanthines dilate the small veins has been well established experimentally.¹²⁻¹⁴ This effect would cause a sidetracking of part of the circulating blood, thus temporarily relieving the load on the failing heart. Such an effect could well account for a decrease of venous pressure and an acceleration of the circulation.

In the older literature there are found reports that the xanthines increase the capillary permeability for water, thereby causing a transudation of fluid from the blood into the tissues. This process would tend to reduce the plasma volume which is usually increased in congestive heart failure¹⁵ and bring on the phenomena which we have observed. However, the reports concerning the increase of capillary permeability due to xanthines are based on inconclusive experimental evidence.¹⁶ Our plasma volume studies indicate definitely that no appreciable amount of fluid leaves the blood stream concomitant with the fall of venous pressure. We were not concerned with the diuretic action which occurs later and may account for such changes as observed by Zak,¹⁷ in which the time factor was not considered.

The question whether or not theophylline stimulates myocardial function and improves the coronary circulation could not be answered by our clinical studies.

SUMMARY

Theophylline aminoisobutanol by intravenous administration causes a fall in venous pressure and a shortening of the circulation time. These effects are the more pronounced the more these functions are elevated above normal.

The venous pressure begins to fall while the injection is still in progress. The effect persists for about sixty to ninety minutes. The circulation time apparently remains shortened for about the same period of time.

The fall in venous pressure is not accompanied by any changes in the plasma volume.

Aminophylline and nitroglycerin produce the same effects.

The venous pressure shows wide fluctuations apart from any drug effect.

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STIMULATION OF THE CENTRAL NERVOUS SYSTEM BY CURARE (INTOCOSTRIN)

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THE increasing use of curare (Intocostrin)* as an adjunct to anesthesia and for other clinical purposes indicates the need for additional information regarding its action in the body. The work of West¹ seems to be the only recent extensive pharmacologic study of curare preparations. The investigation reported herein is a contribution to the toxicology of the particular curare alkaloid now readily available.

Intocostrin is reported by the manufacturers to owe its curare action (interference with the passage of impulses from motor nerves to skeletal muscles at the neuromuscular junction) almost entirely to its content of d-tubocurarine, an alkaloid found in Amazonian curare. This alkaloid has been crystallized and its chemical structure established fairly completely.² It is less potent than curarine, the characteristic alkaloid of curare from British Guiana.

METHODS

Intocostrin (from 0.5 to 10.0 units per kilogram)† was administered to rats, mice, guinea pigs, rabbits, and cats subcutaneously, intramuscularly, intraperitoneally, or intravenously. For all injection routes, the drug was diluted if necessary to keep the volume at least 0.4 c.c. The marginal ear veins of unanesthetized rabbits and the exposed femoral veins of rats were used for intravenous injections. The rats were lightly anesthetized with ether for the exposure of the veins and were allowed to recover to the point of moving spontaneously before the intocostrin was injected. Practically all the injections were made rapidly (within two to five seconds); a few slow (from one to four minutes) intravenous injections into rabbits indicated that the rate of injection did not make a significant difference in the effects. Crystalline d-tubocurarine (6.17 standard intocostrin units per milligram) was dissolved in Ringer's solution and administered in suitable dilution by rapid subcutaneous or intramuscular injection to mice and to a few rats and guinea pigs (from 0.5 to 1.0 mg. per kilogram).

Most of the experiments with intocostrin were done on rats (weighing from 150 to 200 grams). No difference in the susceptibility of the sexes was noted, but it was found that the amount of intocostrin required to kill the rats decreased as their stay in the laboratory was prolonged. The deciding factor in this effect was presumably either age or nutrition, but this was not determined. A homogeneous group of rats was used as far as possible for the sodium amylal series and its control (Table III).

Sodium amylal (from 75 to 100 mg. kilogram, intraperitoneally) was found to produce complete anesthesia in our rats without intocostrin. As the combination of this dose

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†Intocostrin is adjusted by the manufacturers to contain 20 units per cubic centimeter, a unit being measured by the rabbit head drop crossover method. In this method small increments of the drug are injected intravenously at fifteen-second intervals until the rabbit is unable to raise its head under reflex stimulus. An early description of the method is found in Bennett,³ p. 104 and Plate 34. We found that 1 unit per kilogram injected in two to ten seconds into the ear vein of each of two rabbits produced head drop that appeared in a few seconds and lasted a few minutes.

with intocostrin was usually fatal, sodium amytal was used at the minimum level (between 50 and 75 mg. per kilogram) that permitted the introduction of a hypodermic needle without causing the animal to withdraw its leg. Intocostrin (2 units per kilogram) was then injected subcutaneously or intramuscularly. In a few animals in the series, the sodium amytal was not injected until severe convulsions had developed following intocostrin injection.

Oxygen, a mixture of 5 per cent CO_2 and 95 per cent O_2 , or cyclopropane, when used, was delivered at a rate of 1,000 c.c. per minute by an anesthesia gas machine into a 2 liter jar in which a rat was placed. Cyclopropane was started in the proportion of 3 parts to 7 parts of oxygen and then was reduced to the concentration required to maintain light anesthesia. Artificial respiration was administered manually or by breathing into a tube placed over the animal's nose.

Neostigmine methyl sulfate (from 0.5 to 0.75 c.c. 1/4000) was injected intramuscularly into two rats.

RESULTS

Effects of Intocostrin and d-Tubocurarine Chloride.—Preliminary injection of intocostrin into frogs confirmed the results of workers with other curare preparations by producing muscular relaxation when the injection was made into the lymph sac and stimulation and tonic convulsions when the injection was made into the cerebrospinal canal through the foramen magnum.

The mammals injected with either intocostrin or d-tubocurarine chloride manifested: (1) weakness, flaccidity, decrease of normal activity, paralysis (regarded as signs of curarization) and (2) with the same or a larger dose, ties, tremors, convulsions (signs of nervous stimulation). All species showed both stimulation and curarization but to varying degrees; muscular relaxation was more prominent in rabbits and cats, nervous stimulation in rats and mice. The course of the poisoning following subcutaneous or intramuscular injection in rats will be described, and then the variations observed with different species or modes of injection will be pointed out. The results of all of these experiments are summarized in Table I.

Small subcutaneous or intramuscular doses in rats (from 0.5 to 0.75 units per kilogram) produced weakness, trembling, and marked decrease of spontaneous movements but hyperreactivity to external stimuli. Rats given 1 unit per kilogram exhibited gnashing of teeth, facial ties, twitching of back muscles, and spasms of respiratory muscles. The animals recovered from these subconvulsive doses in varying short lengths of time and showed no apparent after-effects.

Generalized convulsions of clonic-epileptiform type were produced in all rats given 1.25 or more units per kilogram. The convulsions were preceded by incoordination in walking, generalized trembling and weakness, decreased spontaneous activity, and the other effects observed with smaller doses. A characteristic convulsion began at the mouth, travelled up to the ears and eyes, and thence, like a shudder, down the back; the tail was drawn slightly dorsally. The accompanying or succeeding violent clonic movement of the whole body resulted in some animals in leaps of five or six inches upward and from one to twelve inches forward. The convulsions seemed to arise spontaneously or in response to almost any external stimulus, no matter how slight. An individual

convulsive seizure was occasionally introduced by a tonic phase. Each seizure lasted from a few seconds to a minute. The generalized convulsions were succeeded by a stage of localized muscle spasms and ties. The ability to move in response to external stimuli was not lost at any time in nonfatal cases, nor in 10 per cent of the fatal cases until the very end. In most of the fatal cases, it was retained through the first violent convulsions but disappeared in the asphyxial stage.

The time-course of the poisoning with convulsive subcutaneous or intramuscular doses in rats was as follows: No matter how large the dose, convulsions seldom started within thirty seconds after the injection. The average time to the first convulsion was about three minutes, whether the animal was left free to run about or was confined to a small area. Trembling began about one minute before the first convulsion. Individual convulsions were no more violent in the fatal than in the nonfatal cases, but they were more numerous; the stage of generalized convulsive seizures lasted on the average six minutes. With a nonfatal dose convulsions were followed by recovery in forty-five minutes to two hours. With a fatal dose respiration ceased on the average fifteen and one-half minutes after the injection, and the heart beat was not palpable two minutes later unless artificial respiration was instituted.

Respiration appeared to be normal in rate, depth, and character until the onset of convulsions or even during mild convulsions. With the appearance of violent and frequent convulsions (that is, three seizures per minute), apneusis occurred during the convulsions. Cyanosis was not visible around the mouth or in the paws until this stage was reached, but then it soon became marked, in spite of normal or deep-labored breathing between convulsions. Typical asphyxial gasps eventually developed, and in fatal cases death was due to asphyxia.

The subcutaneous or intramuscular dose required to kill 95 per cent of the rats was 2 units per kilogram. It appeared to be the same whether the drug was given all in one injection or in fractional injections following each other within a few minutes. Although rapidly repeated doses were markedly cumulative, rats given a convulsive, subfatal dose on three successive days seemed entirely normal as to gait, appetite, motor activity, nervous system reactivity, and escape patterns.

The effects of a given dosage of interoestrin were apparently the same after intraperitoneal injection (seven rats) as after subcutaneous or intramuscular injection. With intravenous injection (twenty-six rats), however, the dosage required for any given effect was smaller (Table I), and the effects appeared more promptly. Apneustic breathing began almost immediately after intravenous injection, and there was time for only a few ties and a few tonic movements before death occurred (sometimes within one minute) in fatal cases. Opisthotonus was regularly observed with intravenous injection, in addition to the signs of central nervous stimulation seen with subcutaneous and intramuscular injection. That the ether anesthesia preceding intravenous injection affected the results observed is not unlikely.

The effects of intocostarin in guinea pigs and mice were substantially the same as in rats, except that (1) larger doses were needed to produce the effects (Table I) and (2) the time to the appearance of the signs of poisoning was longer. Subcutaneous injection into mice resulted in poor absorption and few symptoms, and somewhat larger intramuscular doses were required in mice than in guinea pigs. Intramuscular injection of intocostarin into two cats resulted in greater muscular relaxation and less marked stimulation than in rats, mice, and guinea pigs. The cats survived until six to eight units per

TABLE I. A. SUSCEPTIBILITY OF DIFFERENT SPECIES TO INTOCOSTRIN; B. SUSCEPTIBILITY OF MICE TO CRYSTALLINE D-TUBOCURARINE CHLORIDE CONTAINING 1 STANDARD INTOCOSTRIN UNIT PER 0.162 MG.

	DOSAGE UNITS PER KILOGRAM									
	0.5	0.75	1.0	1.25	1.50	2.0	3.0	4.0	5.0	6.0 OR MORE
A. Intocostarin										
Rats										
Subcutaneous or intramuscular	C 0/2 F 0/2	C 0/2 F 0/2	C 0/6 F 0/6	C 2/2 F 0/2	C 5/5 F 1/5	C 40/40 F 38/40				
Intraperitoneal					C 2/2 F 0/2	C 5/5 F 5/5				
Intravenous	C 1/5 F 1/5		C 2/8 F 1/8	C 4/4 F 4/4	C 4/4 F 4/4	C 5/5 F 5/5				
Guinea pigs										
Subcutaneous or intramuscular						C 2/2 F 0/2	C 3/3 F 3/3			
Cats										
Intramuscular						C 0/2 F 0/2		C 0/1 F 0/1		C 2/2 F 2/2
Rabbits										
Intramuscular						C 0/2 F 0/2		C 0/1 F 0/1		C 2/2 F 2/2
Intravenous			C 0/2 F 0/2	C 7/7 F 4/7	C 3/3 F 2/3	C 3/3 F 3/3				
Mice										
Intramuscular			C 0/2 F 0/2			C 0/2 F 0/2	C 3/4 F 2/4	C 3/3 F 1/3	C 3/3 F 3/3	
B. d-Tubocurarine										
Mice										
Intramuscular							C 2/5 F 1/5		C 3/3 F 3/3	C 3/3 F 3/3

Under each dosage is entered the ratio (C) of the number of animals exhibiting convulsions to the number of animals given that dosage and the ratio (F) of the number of animals killed to the number given that dosage.

kilogram were administered. Rabbits were somewhat more resistant than cats to intramuscular intocostarin, from eight to ten units per kilogram being the fatal dose. Rabbits manifested few, if any, signs of stimulation with this route of administration until marked asphyxia developed.

Intravenous administration, however, produced in rabbits qualitatively the same effects as subcutaneous or intramuscular administration in rats, provided a sufficient dose was given. With 1 unit per kilogram intravenously, marked

muscular relaxation, as seen in the head drop method for the standardization of intocostrin, was accompanied by no signs of stimulation, and such relaxation was invariably the earliest effect of larger (stimulant) doses. With 1.25 units per kilogram the relaxation was noted in eight to thirty seconds in the seven rabbits given this dose and was followed in another ten to one hundred seconds by cyanosis with respiratory disturbance, muscular twitchings, increased excitability, and clonic convulsions, especially severe when provoked by stimulation. Convulsive movements were noted in some of these animals before cyanosis, as in rats; more often a slight degree of cyanosis was evident before or at the same time as signs of stimulation. The cyanosis became prominent with the onset of violent convulsions. As shown in Table I, the fatal intravenous dose for rabbits was about the same as the fatal subcutaneous or intramuscular dose for rats, but the rabbits died somewhat more quickly (usually within ten minutes).

Crystalline d-tuboenerarine was injected intramuscularly into eleven mice, and the results are embodied in Table I(B). Expressed in units, the convulsant and fatal activities of this form of the alkaloid do not differ significantly from those of intocostrin in mice. The crystalline form produced qualitatively the same effects, including the stimulant ones, at about the same times as intocostrin in itself in mice and in a few rats and guinea pigs into which it was injected subcutaneously or intramuscularly.

Antagonists to Intocostrin.—Various efforts to antagonize the effects of intocostrin and the results of these efforts are presented in Tables II and III.

Artificial respiration instituted after the failure of normal respiration prolonged the lives of fatally poisoned rats and rabbits and, if continued long enough to permit detoxification of the drug by the body, would presumably have saved them (Table II). Spontaneous respiration was restored at intervals in these animals, and convulsive movements or frank convulsions occurred during these intervals. All six of the animals given artificial respiration died, as did ten rats to which oxygen or 95 per cent O_2 -5 per cent CO_2 was administered both before and after the intramuscular injection of intocostrin (2 units per kilogram). Although administration of the gas was begun some time before the respiration was affected, the course of the poisoning was not modified in the slightest by this treatment (Table II).

Into two rats given the fatal dose of intocostrin by intramuscular injection (2 units per kilogram), neostigmine was injected a few minutes after the onset of violent convulsions, as soon as the respiration became abnormal (Table II). Normal respiration was restored promptly, and the animals were able to get up within ninety seconds and to struggle forward. Trembling, mild convulsions with movement, and violent convulsions following external stimulation continued for an hour after the neostigmine was injected, decreasing in intensity with time. The animals survived.

The indications that central nervous stimulation participated in the toxic effects of intocostrin suggested the use of a central nervous depressant to antagonize these effects. Sodium amytal was tried and found to save the lives of 60 per cent of the rats subsequently given the subcutaneous or intramuscular

TABLE II. RESULTS OF VARIOUS FORMS OF TREATMENT OF INTOCOSTRIN POISONING

TREATMENT	ANIMALS	DOSAGE INTOCOSTRIN (UNITS PER KILOGRAM)	RESULTS	REMARKS
Artificial respiration after failure of normal respiration	Rats	2	C 4/4 F 4/4	Spontaneous respiration restored for brief intervals by treatment; convulsions occurred during these intervals; death followed omission of treatment from twenty to thirty minutes after artificial respiration was first administered
	Rabbits	125	C 2/2 F 2/2	
100% O ₂ or 95% O ₂ -5% CO ₂ Before and after intocosttrin administered	Rats	2	C 10/10 F 10/10	Course of poisoning exactly as in untreated rats
Cyclopropane (0-30% in O ₂) Before and after intocosttrin administered	Rats	1.5	C 0/1 F 1/1	Concentration of cyclopropane 30% initially, decreased after anesthesia produced; convulsions appeared whenever anesthesia was sufficiently light; level of anesthesia varied as required to prevent convulsions on the one hand or marked depression of respiration on the other; spontaneous respiration was maintained until death in fifteen to eighty minutes
	Rats	2	C 0/3 F 3/3	
Immediately after intocosttrin administered	Rats	2	C 0/3 F 3/3	
From 5 to 20 minutes after intocosttrin following onset of violent convulsions	Rats	2	C 0/2 F 2/2	
Neostigmine methyl sulfate (1/1000) Injected intramuscularly in small portions (total 1 mg. per kilogram) following onset of violent convulsions and impaired respiration	Rats	2	C 2/2 F 0/2	Convulsions or other signs of stimulation persisted for an hour after neostigmine, decreasing in intensity with time

See Table III for results with sodium amytal. Symbols as in Table I. Intocosttrin was administered subcutaneously or intramuscularly to rats, intravenously to rabbits.

TABLE III. PROTECTION OF RATS GIVEN INTOCOSTRIN (2 UNITS PER KILOGRAM SUBCUTANEOUSLY OR INTRAMUSCULARLY) BY INJECTION OF SODIUM AMYTAL (FROM 50 TO 75 MG. PER KILOGRAM INTRAPERITONEALLY)

	TOTAL NUMBER OF RATS	PER CENT SHOWING CONVULSIONS			PER CENT KILLED	AVERAGE TIME TO CESSATION OF RESPIRATION IN FATAL CASES	
		NONE	MILD	SEVERE		MINUTES	STANDARD ERROR
Intocosttrin alone	40	0	0	100	95	15.6	±1.4
Intocosttrin preceded by sodium amytal	50	67	33	0	40	30.3	±6.1
Intocosttrin followed by sodium amytal	10	100 after sodium amytal	-	100 before sodium amytal	100	-	-

dose of intocostirin (2 units per kilogram) fatal to 95 per cent given no depressant (Table III, column 6). Statistical analysis shows the difference between the per cent mortality in the control series and that in the amytal-treated series to be highly significant. All of the survivors lived and were normal for the fortnight of observation following the experiment.

The rats given sodium amytal either before or after intocostirin all manifested complete muscular relaxation early in the poisoning, in contrast to animals given no depressant in which complete muscular relaxation appeared terminally only, if at all. Death, when it occurred in the amytalized rats, followed a prolonged period of flaccid paralysis and nonresponsiveness. In rats given the depressant after convulsions had developed, the convulsions became less violent and finally ceased, but the animals nevertheless died. Possibly they lived somewhat longer than if they had not received sodium amytal. The ability of the depressant to prolong life is more certainly shown in the series of animals injected with sodium amytal before intocostirin; the rats in this series, which were not saved by the sodium amytal, lived on the average twice as long after the intocostirin was administered as the unprotected rats did (Table III, column 7).

Because intocostirin is known to be useful clinically in anesthesia with cyclopropane, an attempt was made to protect rats against a fatal dose of intocostirin by means of this anesthetic (Table II). It was found possible to keep the rats alive for over an hour and free from convulsions during this time, whether cyclopropane was started before or after intocostirin was administered. However, all of the animals eventually died under the anesthetic or following convulsions which developed when the anesthetic was removed. Experience with the method and consequent improvements in technique might show cyclopropane to be an effective antidote to intocostirin.

DISCUSSION

Interference with the passage of impulses from nerves to muscles, typical curare action, is taken to be the pharmacodynamic action of d-tubocurarine (and therefore of intocostirin which contains tubocurarine) that produces weakness, muscular relaxation, and paralysis in mammals. Involvement of the respiratory muscles in this effect certainly accounts in part for the fatal asphyxia, as shown by the lifesaving ability of neostigmine in our rats and in the rabbits given d-tubocurarine in fatal doses intravenously (1.5 standard intocostirin units per kilogram) by Koppanyi and Vivino.⁴ These observations with neostigmine are in agreement with much earlier work on anticholinesterases as antidotes to curare. The curarization is obviously far from complete in all the mammals used in our experiments. In spite of the current view that the respiratory muscles are the last to be affected by curare, curarization of the respiratory muscles does not seem to be less complete than that of the other skeletal muscles. The two following reports from the literature on curarine in rats are pertinent in this connection. Blume⁵ was unable to achieve neuromuscular paralysis (tested by electrical stimulation of sciatic nerves) even with near-fatal doses.

West^{1, p. 86} found that with electrical stimulation of the nerves conduction from the phrenic nerve to respiratory muscles might outlast conduction from the sciatic nerve to leg muscles or might disappear before the latter.

Stimulation of the central nervous system, a second, much less familiar action of curare, is probably also a factor in the action of d-tubocurarine or intocostarin in mammals. Phenomena attributed to central stimulation (muscular spasms, convulsions) have been observed consistently following direct application of curare preparations to the central nervous system (frogs, dogs).^{6, 7} That the underlying mechanism is actually stimulation of nervous tissue has been established by Eccles' recent report⁸ on the effect of curarine on the spinal cord and roots isolated from the frog. Under the influence of curarine in a concentration twice as great as necessary to block neuromuscular transmission, both the synaptic potential of the cord and the discharge of impulses set up by a single dorsal root volley were found to be prolonged. With much higher concentrations continuous electrical activity occurred spontaneously. The actions of curarine on the electrical behavior of the cord therefore resemble those of strychnine and are the reverse of curarine effects at sympathetic ganglia and at junctions between motor nerves and skeletal muscles. Our preliminary experiments with intocostarin injected into the cerebrospinal canal of frogs demonstrate that intocostarin like other curare preparations stimulates the central nervous system when applied directly to it.

Signs of stimulation have also frequently been observed when curare was administered by methods other than direct application to the central nervous system. Blume produced convulsions and other signs of stimulation in rats, mice, guinea pigs, and, in a milder degree, even in cats by the subcutaneous or intravenous administration of curarine or calabash-curare.⁵ West observed stimulation of several mammalian species with various curare preparations and came to the conclusion that central nervous excitation is an action of certain forms of curare, although apparently not of curarine or d-tubocurarine.¹ That tubocurarine nevertheless actually possesses stimulant activity is strongly indicated by the recent work of McIntyre, Dunn, and Tullar.⁹ They report increased electrical activity of the brains of dogs given this alkaloid intravenously. The increase, although "transient," appears to be considerable and may affect either voltage or frequency. The succeeding period of depressed activity in these animals recalls the observations of Pick and Unna¹⁰ on the brains of pithed frogs given d-tubocurarine into the lymph sac. They observed inhibition and suppression of brain electrical activity with this and other curarizing substances; however, inasmuch as strychnine and picrotoxin failed to have any stimulant effect in similar frog experiments, these observations do not contradict the view that intocostarin stimulates the central nervous system of mammals directly.

It may be argued that the signs of central stimulation observed following the administration of curare preparations by other routes than direct application to the central nervous system are in fact due not to drug action on this system but to the production of asphyxia by the curarization of respiratory

neuromuscular junctions. This interpretation, although found in the literature,^{11, 12} is difficult to defend in view of (1) the stimulant activity of directly applied curare, (2) the limited degree to which classical curarization occurs in mammals, and (3) the presence of stimulation in some cases in the absence of overt asphyxia.

That direct central nervous stimulation plays a significant role in the action of intocostrin administered in large enough dosage by any route to mammals is indicated by (1) the ability of sodium amytal to prolong or save the lives of rats given a fatal dose of this form of curare, (2) persistence of signs of central nervous stimulation in rats after antagonism of the peripheral neuromuscular effects of neostigmine, and (3) lower fatal dosage for species responding to the drug with marked stimulation (rats, guinea pigs) than for species responding with marked relaxation (cats, rabbits). Some of the species difference is associated with difference in rate of absorption of the drug from the tissues, but the difference in the size of the fatal intravenous dose for rats and rabbits, for instance, is probably associated with the greater ease of producing convulsions in the former.

Since the protective effect of neostigmine affords evidence that neuromuscular depression also plays a significant role in the toxic action of intocostrin, it is assumed that both central stimulation and neuromuscular depression contribute to the intocostrin asphyxia fatal to mammals. The asphyxiating power of depression of respiratory neuromuscular junctions is obvious. Central stimulation by producing convulsions would greatly increase the oxygen used by the body (perhaps three- or fourfold if the convulsions were equivalent to severe exercise) at a time when the respiratory apparatus was so incapacitated by the peripheral effect of the intocostrin as to be hardly able to meet the basal oxygen requirements. Moreover, convulsive waves of activity in the brain doubtless interfere with the orderly transmission of respiratory impulses and therefore lead to inadequate respiratory movements or respiratory arrest. Any agent that prevented convulsions would therefore decrease the degree of asphyxia, and anyone of many central depressants might be expected to protect animals against a fatal dose of intocostrin.

Koppányi and Vivino⁴ observed that ephedrine and tyramine, although themselves useless as antidotes to d-tubocurarine, when combined with physostigmine or neostigmine, protected rabbits against 1.33 to 2 fatal doses of the curare alkaloid. Physostigmine or neostigmine alone had no protective value against more than one fatal dose (1.5 units per kilogram).⁴ The mechanism by which the long-lasting sympathomimetic agents produce their effect is unknown.

The classification of curare convulsions as strychninelike by various workers seems to us incorrect for mammals; McGuigan's⁷ statement that the convulsions resemble those produced by picrotoxin more than those produced by strychnine agrees with our experience. In McGuigan's experiments as in ours and in all others described in any detail in the curare literature, the character of the convulsions indicates that, in contrast to strychnine, the curare alkaloids

produce convulsions in mammals by stimulation at a central nervous system level higher than the spinal cord.

Our experimental results reinforce the frequent but not superfluous warning against the use of intocostarin in patients without having immediately available effective means (artificial respiration, neostigmine) for combating respiratory disturbances. In addition, our results suggest that some means of producing central depression should also always be available when intocostarin is to be used clinically. Although it is clear from the size of the dose recommended for clinical use (from 1.1 to 3.3 units per kilogram, intravenously or intramuscularly) that man must be relatively insensitive to the stimulant effects of d-tubocurarine (see also West,^{1, p. 52}) signs of stimulation (muscle twitchings, reflex activation) have been reported with intocostarin in unanesthetized patients¹³ and incompletely anesthetized patients.¹⁴ That these signs are not entirely asphyxial is shown by clinical success in controlling them with increased anesthesia. Certain it is that such signs do not indicate the need for more intocostarin. Griffith believes that in large doses intocostarin has some central action in man, but he does not specify the nature of the action.¹⁵

In addition to overcoming any central stimulation produced by intocostarin, the use of a central depressant has the advantage of increasing the degree of muscular relaxation resulting from a given intocostarin dose. In animals, muscular relaxation with intocostarin is incomplete unless a central nervous depressant is used; evidently central depression makes an important though obscure contribution to "curarization" of skeletal muscle by intocostarin. In unanesthetized patients, likewise, complete muscular relaxation is not ordinarily observed as the result of the peripheral action of intocostarin, although it has been reported with large intravenous doses causing unconsciousness.¹² Cullen, who was unable to obtain sufficient abdominal muscle relaxation to permit satisfactory examination of a patient without premedication even with the intravenous injection of an amount of intocostarin that resulted in respiratory paralysis,¹² regards properly conducted inhalation anesthesia as essential for the successful and safe production of complete muscular relaxation with intocostarin.¹⁶ Mallinson¹⁵ considers anesthesia as deep as the second plane of the surgical stage requisite for maximum abdominal relaxation when intocostarin is used.

For two reasons, therefore, to antagonize any central stimulation and to contribute towards muscular relaxation, means for depressing the central nervous system would appear to be potentially valuable whenever intocostarin is to be used. Our experiments do not indicate which would be the most useful depressants for this purpose. They do however suggest that sodium amytal should be used with great caution, if at all, in view of the toxicity to rats of the combination of sodium amytal with intocostarin. The cause of the toxicity was not investigated; it may be related to one or several of the known side actions of amytal in the body. For surgical operations, intocostarin is frequently combined with one of the inhalation anesthetics or with sodium pentothal. Possibly one of these should be the depressant available for use if needed with intocostarin in other cases. However, two of these depressants have been shown to possess

curariform activity (ether in considerable degree, sodium pentothal slightly)¹¹ and so are not unobjectionable. The known central stimulant action of morphine makes it unsuitable likewise.

SUMMARY

1. Intocostrin or crystalline d-tubocurarine chloride, injected in sufficient dosage subcutaneously, intramuscularly, intraperitoneally, or intravenously into rats, mice, guinea pigs, rabbits, and cats, produces hyperexcitability and clonic convulsions in addition to partial curarization.

2. Signs of central nervous stimulation are more conspicuous in some species than others and are shown by rats without signs of asphyxia. Species (rats, guinea pigs, mice) manifesting much stimulation are killed by smaller doses of the drug than species (rabbits, cats) manifesting more curarization.

3. With fatal doses, death in all these animals is due to asphyxia, attributed in part to curarization of respiratory neuromuscular junctions peripherally and in part to stimulation centrally. The asphyxia may be relieved by neostigmine (peripheral action) or by sodium amytal (central action). Sodium amytal protects 60 per cent of rats from the effects of a dose of intocostrin (2 units per kilogram) fatal to 95 per cent of untreated rats. The other 40 per cent of rats given sodium amytal prior to intocostrin live twice as long after the intocostrin injection as those not given sodium amytal.

4. Central nervous depressants (sodium amytal, cyclopropane), decrease, abolish, or prevent intocostrin convulsions. Reduction of asphyxia by oxygen administration or artificial respiration is less, if at all, effective in controlling convulsions.

5. The conclusion from these observations that central nervous stimulation is one of the pharmacodynamic actions of intocostrin is in agreement with the conclusions of other workers using other curare preparations.

6. The flaccidity and decrease of spontaneous movements manifested by mammals given intocostrin or d-tubocurarine alone do not develop into complete neuromuscular paralysis until just before death. In animals given a central nervous depressant in addition to intocostrin muscular relaxation becomes complete at an early stage of intocostrin action.

7. Certain central nervous system depressants may be useful adjuncts to intocostrin to combat central stimulation as well as to increase muscular relaxation.

The author would like to express her sincere appreciation to Dr. Helen Graham, for her aid and advice in this research.

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HEMOGLOBIN LEVELS IN SPECIFIC RACE, AGE, AND SEX GROUPS OF A NORMAL NORTH CAROLINA POPULATION*

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A STUDY of the distribution of hemoglobin levels in a population may furnish important information regarding its nutritional status. At the same time, the expected frequency of occurrence of different hemoglobin values in a presumably normal population should be delimited in order to indicate those which may have a pathologic basis.

During the years 1940-1945 large population groups in North Carolina were studied to determine their nutritional status. In the course of these surveys a careful estimate of hemoglobin level was made for each of 3,029 persons, 2,168 of whom were white and 861 (28.4 per cent) colored. This racial distribution represents fairly closely that of the area studied and that of the state as a whole. The surveys were conducted in Chatham, Alamance, Orange, and Wayne counties. With the exception of Wayne, all of these counties lie in or near the fall line just west of the coastal plain. The results of the studies have appeared in several publications.¹⁻³ The samples selected for the study comprise families chosen to represent as nearly as possible the population of the county in question.

Wayne County, in the mideastal plain, was the only one in which hookworm infection or malaria might affect the "normality" of the hemoglobin values. Extensive hookworm studies recently made there⁴ showed a light infestation of some 20 per cent of the population. Of the blood films taken throughout the year of study in this county and examined by the malaria section of the North Carolina State Board of Health, only one showed malaria parasites. No other disease was recognized which might have unduly influenced the hemoglobin levels. It may be assumed, therefore, that the population studied provides a fair picture of hemoglobin values for a normal population from this area.

METHOD

Evelyn's method⁵ of determining hemoglobin by the photoelectric colorimeter was employed. Blood samples of approximately 30 c.c. were oxalated when drawn in small bottles with rubber stoppers, and hemoglobin tests were made some two to four hours later. At this time the blood components were remixed by inverting the bottle a minimum of fifteen times, and the pipette was filled from the middle of the bottle. Red cell counts and hematocrit and hemoglobin determinations were then made. One-tenth cubic centimeter of whole blood was added to a flask containing 50 c.c. of distilled water, the pipette being thoroughly rinsed therein. Ten cubic centimeter aliquots, each with one drop of

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concentrated ammonia added and mixed, were examined within ten minutes in the Evelyn colorimeter with 540 filter and 10 mm. aperture and dim light. The galvanometer readings were converted into L values from Evelyn's tables. The L values were multiplied by 100/2.58 to obtain estimates of grams of hemoglobin per 100 c.c.

STATISTICAL TREATMENT

The examined population was subdivided by color and sex into two age groups, differentiating persons under 12 from those who were 12 and over. There are, therefore, eight subgroups exhibiting certain differences for consideration. In Figs. 1 and 2 are shown graphically the distribution of hemoglobin values for each subgroup. To facilitate comparison the frequencies pertaining to each distribution have been converted to percentages of the total number of persons in the subgroup. These are plotted on the vertical scale of the histogram. On the base scale the amounts of hemoglobin in grams per 100 c.c. of blood are entered for the mean of the group and for various multiples of the standard deviation.

In Table I are listed the values of the means and standard deviations for each subgroup, together with their standard errors. Also included in Table I are the limiting hemoglobin values for each distribution, between which half of

TABLE I. DISTRIBUTION CONSTANTS OF HEMOGLOBIN LEVELS FOR WHITE AND COLORED POPULATIONS BY AGE AND SEX, AND WITH LIMITS BETWEEN WHICH ONE-HALF AND NINETEEN-TWENTIETHS OF THE OBSERVATIONS MAY BE EXPECTED TO FALL

		WHITE		COLORED	
		NUM- BER	GRAMS OF HEMO- GLOBIN PER 100 C.C.	NUM- BER	GRAMS OF HEMO- GLOBIN PER 100 C.C.
Mean					
Under 12	Boys	357	12.67 \pm 0.046	193	11.89 \pm 0.078
	Girls	362	12.54 \pm 0.046	188	12.13 \pm 0.062
12 and over	Men	611	14.25 \pm 0.049	212	13.76 \pm 0.086
	Women	838	12.87 \pm 0.043	268	12.16 \pm 0.087
	Women (fitted curve)	796	13.08	236	12.64
Standard deviation					
Under 12	Boys		0.867 \pm 0.032		1.082 \pm 0.055
	Girls		0.874 \pm 0.032		0.845 \pm 0.044
12 and over	Men		1.209 \pm 0.035		1.249 \pm 0.061
	Women		1.254 \pm 0.031		1.430 \pm 0.062
	Women (fitted curve)		0.911		0.815
Limits of one-half distribution					
Under 12	Boys		12.09 - 13.25		11.16 - 12.62
	Girls		12.01 - 13.11*		11.56 - 12.70
12 and over	Men		13.43 - 15.07		12.92 - 14.60
	Women		12.02 - 13.72		11.20 - 13.12
	Women (fitted curve)		12.48 - 13.70		12.09 - 13.19
Limits of nineteen-twentieths of distribution					
Under 12	Boys		10.97 - 14.37		9.77 - 14.01
	Girls		10.95 - 14.17*		10.47 - 13.79
12 and over	Men		11.88 - 16.62		11.31 - 16.21
	Women		10.41 - 15.33		9.36 - 14.96
	Women (fitted curve)		11.30 - 14.88		11.04 - 14.24

*First individual (hemoglobin, 6.5 Gm.) omitted.

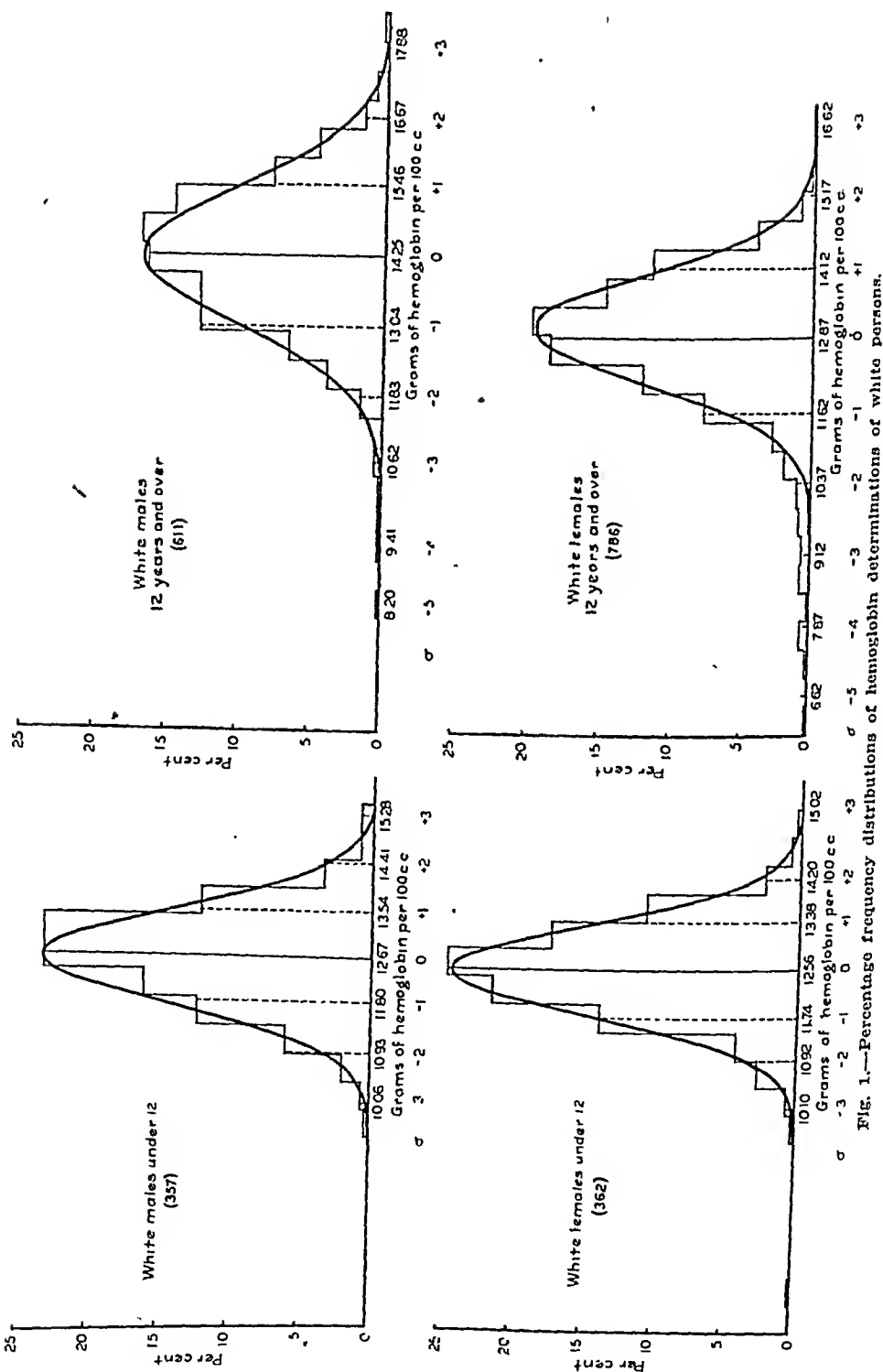


Fig. 1.—Percentage frequency distributions of hemoglobin determinations of white persons.

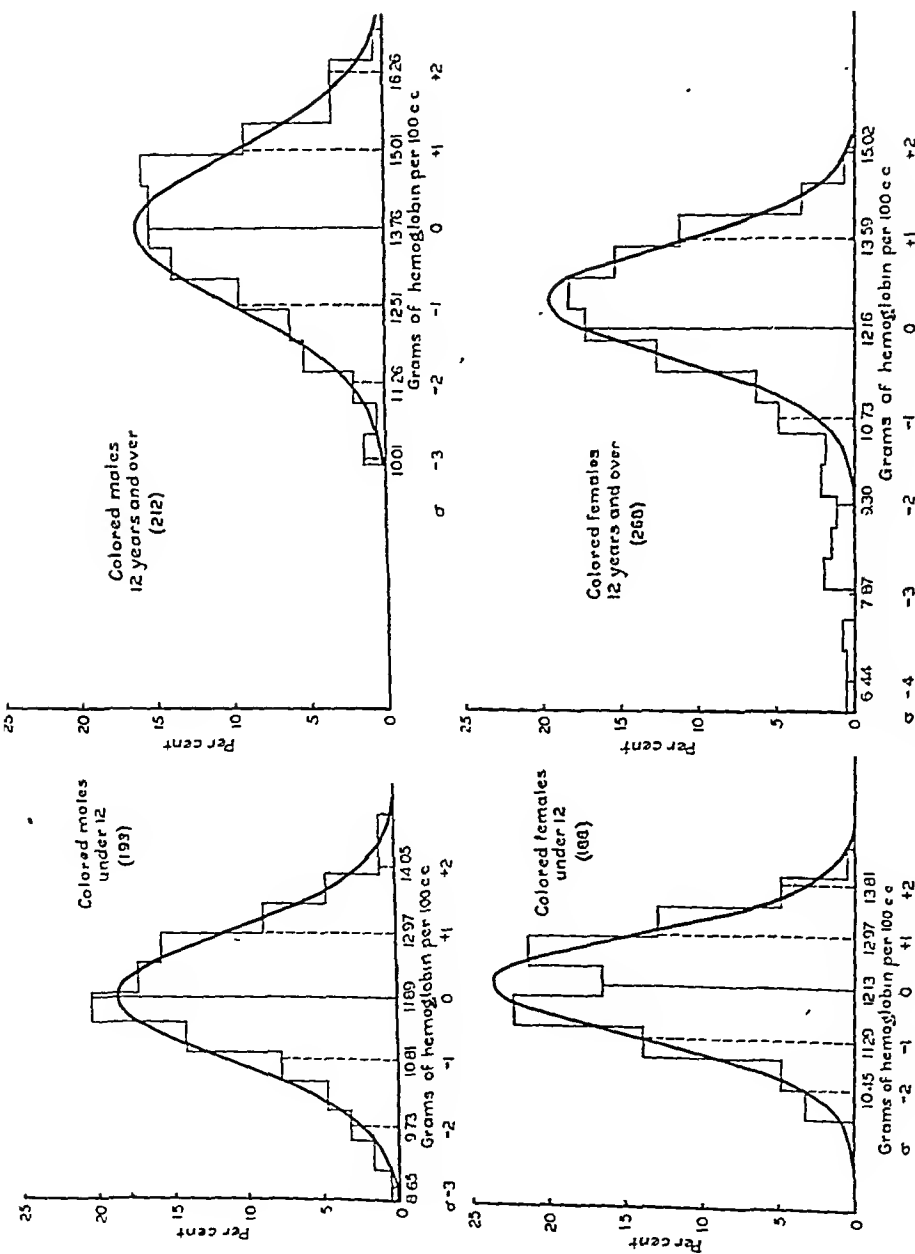


FIG. 2.—Percentage frequency distributions of hemoglobin determinations of colored persons.

the observed values may be expected to fall, as well as the limits between which nineteen-twentieths of the expected values will lie. Individual observations falling outside the latter limits would be rare and therefore under suspicion of being abnormal.

The histograms for six of the distributions shown in Figs. 1 and 2 are fitted with normal frequency curves having the means and standard deviations of the observed distributions. It is apparent that these curves are good expressions of the observed occurrence of hemoglobin values and may be used to express the probability of obtaining other observations in such groups between any given values. It is obvious, however, that when such curves are applied to the data for the two groups of women, although they describe the distribution of the great majority of the observations, they fail to take into account a number with very low values. Among white adult females 42 of the 838 individuals (5 per cent) fall below and outside the normal curve fitted to the bulk of the observations, and among colored women 32 of 268 (12 per cent) fall below the lower limit set by the fitted curve.

None of the usual frequency distributions of the Pearsonian series gave an adequate picture of the observations pertaining to the two groups of women. Finally, normal frequency curves were fitted to approximately three-quarters of the observations at the upper end of the distribution by the method of incomplete moments⁹ and the curves then extended through the lower ends of their ranges. As indicated in Figs. 1 and 2, there is an excess of observations for each of the two groups over the expected number at the lower end of the hemoglobin scale.

In Table I two sets of means and standard deviations are given for each group of women. Those derived from all the observations are entered first and those from the fitted curve, second. The means and standard deviations plotted in Figs. 1 and 2 are the ones pertaining to all observations.

DISCUSSION OF RESULTS

It appears that, in general, the hemoglobin values encountered in the subgroups of this surveyed population can be expressed in terms of a so-called normal frequency distribution. This is strictly true for men and for children of both sexes with the exception of one girl with 6.5 Gm. of hemoglobin per 100 c.c., which was completely outside the range of expected values. From 90 to 95 per cent of women have values which are well described by the normal curve, with the remaining observations falling below the expected range. The fact that this divergence is confined, with one exception, to women suggests that the apparent deficiency is linked with the reproductive processes, possibly with the menstrual cycle. At any rate, low hemoglobin values may be encountered occasionally in women without arousing the suspicion of an acute disease process. In a survey of hemoglobin levels in Great Britain in 1943⁷ the values of 1 per cent of men were found to fall below 80 (Haldane-Gowers hemoglobinometer reading), while those of 6 per cent of single women and 10 per cent of married women fell below this level, which tends to confirm the observations reported in this paper.

In Table I it is indicated that the average hemoglobin values for white persons in all groups are higher than the corresponding ones for colored persons, and the difference in each instance is statistically significant. Sex differences in average level are negligible for children, but men have considerably more hemoglobin than do women, even if the very low values pertaining to the latter are omitted. Men have a higher average level than do boys, but the average for women is only slightly higher than that for girls.

The dispersion of the observations, as measured by the standard deviation, is greater among adults, both males and females, than it is among children. The scatter of the values under the fitted curves for women, however, is similar to that for girls. If the fitted curve may be considered, an expression of the distribution of hemoglobin values not affected by the factor or factors tending to pull down the level, there is little difference between the distributions of girls and women or between those of boys and women. Men, however, present definitely higher levels which are accompanied by a wider scatter of the individual observations.

Again it may be concluded from Table I that only rarely will a normal white child be encountered in such a population with less than 11 Gm. of hemoglobin per 100 c.c. of blood, a white man with less than 12 Gm. or a white woman with less than 10.4 Gm. Corresponding values for a colored population would be from 0.5 to 1 Gm. less.

TABLE II. MEAN DAILY INTAKE OF IRON IN THE DIET (FOUR NORTH CAROLINA COUNTIES, 1940-1945)

LOCATION AND DATE	NUMBER OF INDIVIDUALS		MILLIGRAMS OF IRON	
	WHITE	COLORED	WHITE	COLORED
Chatham County, 1940-1941	244	73	8.6	6.3
Wayne County, 1941-1943	933	453	9.4	8.8
Alamance County, 1943-1944	612	139	11.7	11.2
Orange County, 1944-1945	232	0	16.2	
Total	2021	665		

Attempts to raise hemoglobin levels in individuals have not always resulted in a permanent rise, even while curative treatment is being continued.⁵ Furthermore, individual variations within a normal range are to be expected, as has been shown here. Consequently, a person with a low reading, particularly if a woman, must be carefully examined before such a value can be assumed to have a pathologic basis. While menstrual blood loss offers a ready explanation for low hemoglobin levels among women, there is need for more definite information regarding the correlation between the amounts of blood lost and hemoglobin readings before this reason can be accepted as valid.

Dietary factors no doubt influence hemoglobin levels. Variation in copper intake as well as that of protein must affect them. There was no apparent protein deficiency in the group here studied since there were only four individuals with less than 6.0 Gm. of plasma protein per 100 c.c.⁹

Dietary intake of iron may be assumed to be the most important factor affecting hemoglobin levels. During the five and one-half years of the survey,

data on iron intake in the four counties were assembled and are summarized in Table II. In every instance the iron intake fell below the recommended allowance, being approximately one-half the amount in one group. Whether this influenced hemoglobin levels is difficult to determine from the data on hand. A discussion of iron intake with respect to hemoglobin level may be found in previous reports.¹⁻³

The chief sources of dietary iron in the most recently surveyed group (Orange County) were calculated, and the results for persons 15 years of age and over were as follows¹⁰:

FOOD	PER CENT OF TOTAL IRON	
	MEN	WOMEN
Dried beans	24.4	24.7
Enriched bread	11.5	8.4
Eggs	10.1	8.8
Lean pork	6.5	6.8
Milk	4.9	3.4
Dried peas	4.1	3.7
Whole cereal	3.8	6.4
	<u>65.3</u>	<u>62.2</u>

It might be assumed that the fondness of southern people for greens such as turnip tops, mustard greens, and collards, would make these the chief sources of iron. Actually the bulk of iron consumed is contributed by foods which are poorer individual sources but are eaten in larger quantities. Beans were the outstanding source for all groups, contributing about a fourth of the total iron intake. For children, whole cereals were the second source with eggs or milk third.

SUMMARY

This paper presents distributions of hemoglobin levels as determined by the Evelyn photoelectric colorimeter in 2,168 white persons and 861 colored persons in four North Carolina counties. Members of the families examined were living at home and apparently in normal health. The examined population was divided into eight subgroups according to race, sex, and age. The mean grams per cent of hemoglobin ranged between 12 and 14. Means for colored persons were from $\frac{1}{2}$ to 1 Gm. below those for corresponding groups of white persons. Means for women of both races were about 1.5 Gm. below those for men. Differences between means for boys and girls were not great for either race. In general, observed values were well described by normal frequency curves, but among women of each race there was a group comprising from 5 to 10 per cent of the total with hemoglobin values lying definitely below and outside the range of the curve fitted to the main group of observations. Calculated dietary iron intakes for the population under observation was somewhat below that usually recommended as a minimum requirement.

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HEMATOLOGIC SURVEY OF REPATRIATED AMERICAN MILITARY PERSONNEL

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INTRODUCTION

THIS report describes a hematologic survey undertaken to determine the incidence, severity, and type of anemia, as well as the status of the leucocytes and platelets, in repatriated American military personnel from the Far East. It was a part of a general survey carried out at Crissy Annex, Letterman General Hospital, San Francisco, by the Board of Medical Survey of Repatriated American Military Personnel.

A hemoglobin determination (Haden-Hansser) was made on each individual during a general medical examination. On the basis of these determinations, the following day a small group of anemic individuals was selected for a more detailed hematologic study. It is this group of seventy-five individuals which is the subject of a more detailed report in this paper.

The hematologic methods used in the study of the seventy-five selected subjects were those described by Wintrobe.¹ Blood was collected in oxalate bottles containing a 2:3 mixture of potassium and ammonium oxalate. Duplicate red counts were done on each sample of blood. If the two counts did not check within twenty-five cells they were repeated. Hemoglobin determinations were made with a Sahli hemoglobinometer. The hematocrit tubes were spun at 3,000 r.p.m. for thirty minutes in a large International type centrifuge. Such a procedure was found to insure maximal packing under the conditions of this study. Using these methods normal indices were obtained on blood from normal individuals. In experiments using heparin as an anticoagulant, no significant difference in these values was observed. Most of the determinations in these seventy-five subjects were done by one of us (G. E. C.) personally. A few were done by a trained experienced technician under supervision. Serum iron determinations were made using the methods of Kitzes, Elvehjem, and Schuette.² Using this method we have found the range for serum iron in healthy young men to be from 90 to 150 micrograms per 100 c.c.³ This is a somewhat narrower range than that reported by several investigators⁴ and is probably explained by the fact that our group was restricted to young men. The method of Cartwright, Jones, and Wintrobe⁵ was employed for the determination of serum copper. We have found the range for serum copper in healthy young men to be from 90 to 140 micrograms per 100 c.c.³

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CLINICAL HISTORIES

The subjects of this study had been prisoners of the Japanese for periods of from thirty-nine to forty-six months with one exception. This individual had been imprisoned for only nine months. The average duration of imprisonment for the group was 40.1 months. With two exceptions all had been imprisoned in both the Philippines and in Japan.

All individuals gave a history of weight loss varying between 5 and 90 pounds. The average weight loss from the time of capture to the time of release was 37 pounds. This figure, however, does not give a true picture of the degree of weight loss, since many men were below their normal weight at the time of capture and since, due to the dropping of supplies from the air prior to release, many men had gained weight by the time of their actual release.

A history of malaria was obtained in 76 per cent of the group. The number of attacks varied from one to sixty. The average number of attacks per individual affected was ten. Most of these occurred in the early period of the war while the subjects were in the Philippines. During the imprisonment in Japan, in most cases, the malaria subsided spontaneously. None of our subjects had had active malaria within four months prior to this study.

The incidence of certain prominent signs and symptoms of nutritional deficiency as obtained by history is indicated in Table I. Pellagra as evidenced by cheilosis, glossitis, stomatitis, dermatitis, and diarrhea had been present in some 50 to 70 per cent. Several features of this pellagra need comment. The incidence was much greater in the Philippines. Only rarely, if at all, did this symptom complex develop in Japan while the men were sporadically receiving soybeans. The incidence of typical pellagrous dermatitis over the exposed parts was relatively infrequent. This was the case in spite of the fact that the men were constantly exposed to considerable sunlight.

TABLE I. INCIDENCE OF CERTAIN SIGNS AND SYMPTOMS AS OBTAINED FROM HISTORY IN SEVENTY-FIVE REPATRIATED AMERICAN MILITARY PERSONNEL FROM FAR EAST

SYMPTOMS AND SIGNS	NUMBER OF CASES	PER CENT
Cheilosis	54	72
Glossitis	51	68
Stomatitis	59	65
Scrotal lesions	30	40
Pellagrous dermatitis	19	25
Diarrhea	39	52
Edema	58	77
Burning of the feet	35	46
Hyperesthesia of the feet	37	50
Paresthesia of the feet	38	51
Bleeding gums	11	15
Petechiae	1	1
Ecchymosis	2	3
Night blindness	5	7
Photophobia	9	12
Diminution of vision	13	17
Burning of eyes	12	16
Excessive lacrimation	9	12
Weight loss	75	100
Weakness	75	100
Lassitude	75	100

The occurrence of a sealy, sometimes an erythematous weeping scrotal, dermatitis accompanied by extreme tenderness of the scrotum, and in some cases associated with edema, was relatively common. In some cases scrotal tenderness without dermatitis occurred. About 55 per cent of the individuals with a history of glossitis and stomatitis gave a history of scrotal dermatitis. In only one subject did scrotal dermatitis occur in the absence of glossitis and stomatitis. In three cases glossitis and stomatitis developed in the absence of cheilosis. Most generally when glossitis occurred stomatitis also was present. Severe and persistent diarrhea accompanying glossitis and stomatitis was not as frequent as expected. Many, in fact most of the individuals, had short bouts of diarrhea lasting only a few days. In Table I only persistent diarrhea is recorded. No cases of diarrhea resembling sprue were found.

One individual with a severe glossitis gave a history of having received an injection of niacin. Within three hours the burning of the tongue ceased. Another individual with severe diarrhea and dermatitis stated that these symptoms disappeared within two weeks following niacin therapy.

Beriberi was exceedingly prevalent in the group and occurred in both Japan and in the Philippines. A history of wet beriberi was obtained in 77 per cent and a history of dry beriberi in about 50 per cent. Many individuals had had both the wet and the dry types of beriberi, but the most striking feature was that in no individual questioned was a history of both types occurring simultaneously obtained. Often when wet beriberi disappeared symptoms of dry beriberi developed. In other cases, dry beriberi preceded the wet type. Diarrhea was seldom present or severe during the wet phase of beriberi. Massive spontaneous diuresis often took place.

The clinical symptomatology of the dry beriberi was striking. Burning, hyperesthesias, and paresthesias were exceedingly severe. In some camps hundreds of men would walk the floor during the night because of severe pain. Feet were often soaked in ice water, cooled in the snow, or left exposed during cold nights in attempts to alleviate the pain. The feet were so tender that even the lightest touch provoked severe pain. On one occasion a handkerchief was accidentally dropped on the foot of a sleeping soldier. He immediately awoke and cried out in agony. Often just the vibration caused by the passing of an individual within several feet of a soldier with dry beriberi was sufficient to aggravate the pain.

Symptoms suggestive of scurvy were surprisingly infrequent. Fifteen per cent of the subjects gave a history of bleeding gums. In none of these was the bleeding severe. Petechiae were found in only one subject. On examination this individual was found to have a thrombocytopenia. A history of ecchymosis was obtained from only one person in addition to the individual with thrombocytopenic purpura.

Following the intake of a high caloric diet when they were first liberated, in many individuals glossitis, stomatitis, and edema reappeared or became more pronounced.

A history of blood loss was not a prominent feature in these individuals. Six gave a history of bleeding hemorrhoids. In one of these the bleeding was extremely severe. The one individual with thrombocytopenic purpura gave a history of melena. Eleven individuals gave a history of bleeding gums. In each of these the amount of blood lost was extremely small.

DIET

The diet consisted for the most part of rice (white, brown, or red) and "green" soup. The rice was sometimes polished, other times not and was issued in amounts varying from 300 to 700 Gm. per man per day. If a prisoner was not working, or was sick or hospitalized, he was given only 300 to 500 Gm. per day. If working he was given from 500 to 700 grams. In most cases the rice contained rat feces, pebbles, and worms. The most distracting of the three contaminants were the pebbles. Many times whole barley, corn, or soybeans were mixed with the rice. The "green" soup consisted mainly of water to which small amounts of the tops of green vegetables, grass, or green leaves were added. The amount of soybeans available varied from time to time and from camp to camp. To many individuals the soybeans were unpalatable and even under the severest conditions of starvation were refused because of the nausea, vomiting, and diarrhea which resulted from their ingestion. It may be significant, however, that when soybeans were consumed pellagra was not prevalent. The only meat received consisted of a small piece, about 50 to 100 Gm., once or at the most twice a month. During the earlier phases of the war a larger and sometimes more frequent portion was served. During the latter part of the war, in many camps at least, no meat was served. Fish of poor quality was issued one or two times a month at the most. The portions were always small. The only vegetables received were dicon, a Japanese vegetable resembling a combination between a radish and a turnip, carrots, squash, and potatoes. These were served very infrequently. Fresh fruit was not seen. Men resorted to eating snakes, rats, cats, dogs, lizards, frogs, grasshoppers, and snails when these were available, which was infrequent. When animals such as cats and dogs were obtained, not only muscle, but also organs, intestines, eyes, brain, and other portions were eaten. Grasshoppers, if they could be obtained, were boiled to make a soup. For variation they were sometimes roasted. In many cases men received no food for a period of weeks, sometimes only several meals a week, but generally they ate two or three times a day. In all cases the diet was grossly inadequate both in quality and quantity. Food was obtained from outside sources when possible; this opportunity was rare. Men fought over wormy apple cores discovered by the roadside. Red Cross packages were rarely distributed.

PRIOR TREATMENT

The seventy-five subjects had been on a wholesome American diet for from four to ten weeks prior to this study. All had received vitamin supplements either orally or parenterally. Many had received plasma transfusions,

and three had been given whole blood transfusions. Sixteen of our subjects had received from one to twenty liver injections. Ten gave a history of having been treated with iron.

PHYSICAL CONDITION AT THE TIME OF THE SURVEY

At the time of examination all of the men had been released for from two to eight weeks and had been on an adequate diet of from four to ten weeks. The average weight gain for the group since release was 38 pounds. In general, the individuals appeared to be in good condition. About 80 per cent had regained their normal weight. Two characteristic features were present, however; they were a protuberant abdomen and an abnormal distribution of fat. This was present principally over the pelvic girdle and abdomen and was conspicuously absent from the extremities. Twelve individuals still had definite peripheral edema. In thirteen the liver was palpable. Two had a mild glossitis, two an indefinite cheilosis, one follicular hyperkeratosis, one pellagrous dermatitis, and two had residual evidence of optic neuritis. A few individuals had residual evidence of peripheral polyneuritis. One individual had roentgenologic evidence of active pulmonary tuberculosis. This was the only individual in whom an active infection of any nature other than intestinal was demonstrated.

The stools in fifty-seven subjects were examined for parasites. The results were as follows: negative, 25; *Ascaris lumbricoides*, 47; *Necator americanus*, 32; *Endolimax nana*, 14; *Endamoeba coli*, 14; *Endamoeba histolytica*, 5; *Strongyloides stercoralis*, 5; *Trichuris trichiura*, 2; other, 5. In many instances multiple intestinal infestation was present.

In no subject in this group were malarial parasites found in the blood. At the time of the examination all individuals were free of clinical symptoms of malaria, and none gave a recent history of malaria.

HEMATOLOGIC DATA

Incidence of Anemia.—Hemoglobin values below 14 Gm. per cent were found in 52 per cent of the first 1,500 patients seen (Fig. 1). Hemoglobin values less than 14 Gm. per cent were found in 62 per cent of 193 examinees seen during the first week of the survey. In each successive week there was a tendency for the incidence of anemia to diminish (Fig. 2). By the sixth week the incidence had diminished to 35 per cent.

Severity of the Anemia.—The anemia was either mild (from 11 to 14 Gm. per cent hemoglobin) or moderate in severity (from 8 to 11 Gm. per cent hemoglobin) (Fig. 1). Only one case of severe anemia was observed. In Fig. 3 the severity of anemia as demonstrated by hematocrit determinations is presented. The values fell between 27 and 42 c.c. per 100 c.c. In the majority of cases the volume of packed red cells fell between 34 and 40.

Classification of the Anemia.—In 73 per cent of the seventy-five cases studied the mean corpuscular volume was greater than 92 cubic microns, indicating a macrocytic anemia (Table II). In most cases the macrocytosis was mild (from

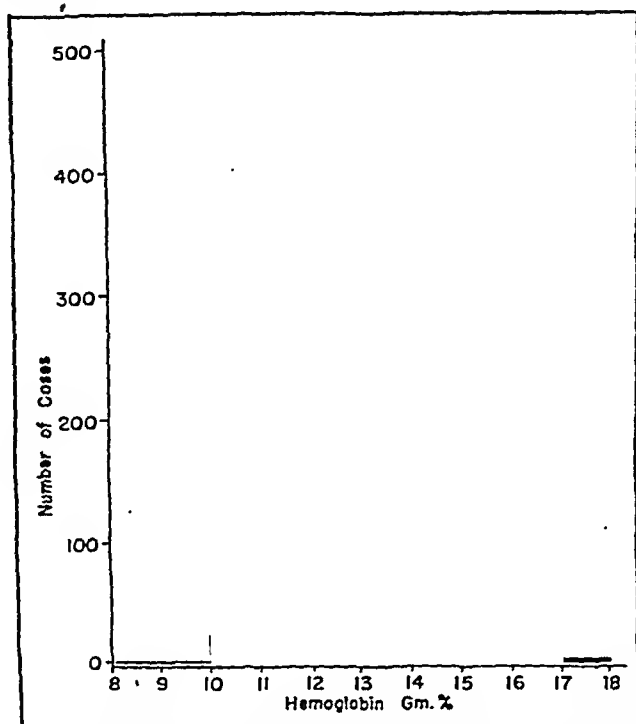


Fig. 1.—Hemoglobin values in the first 1,500 individuals observed.

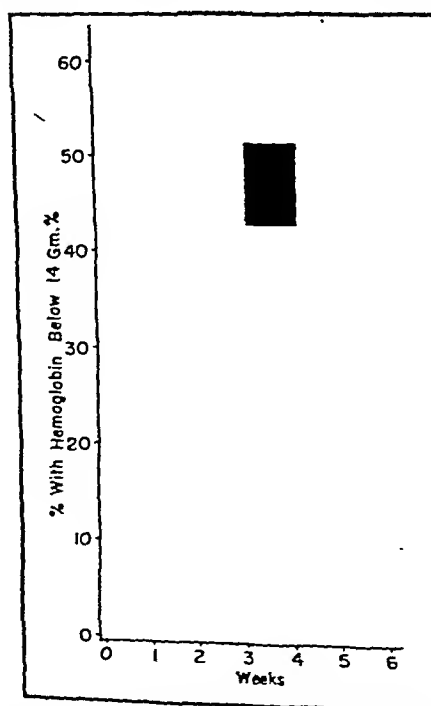


Fig. 2.—The incidence of anemia during each successive week of the survey (3,000 cases).

94 to 104 cubic microns). In twenty cases the mean corpuscular volume was greater than 104 cubic microns, the greatest being 125 cubic microns (Fig. 4). The mean corpuscular hemoglobin for this group was either in the upper range of normal or slightly increased (from 33 to 38 micromicrograms). In two-thirds of the patients studied the mean corpuscular hemoglobin concentration (Fig. 5) fell within the normal range (from 32 to 36 per cent). In the remaining third values were reduced to between 26 and 32 per cent. In the eight patients in whom the mean corpuscular hemoglobin concentration was below 30 per cent, three gave a history of bleeding hemorrhoids, and hookworm infestation was present in two.

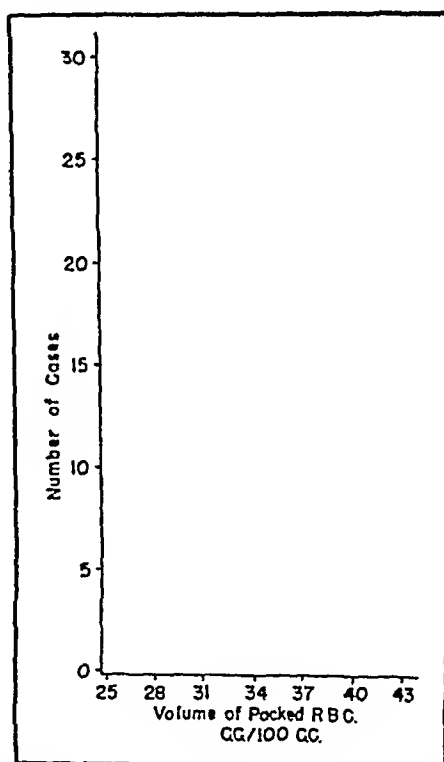


Fig. 3.—The severity of the anemia as measured by hematocrit determinations in the seventy-five subjects studied in detail.

One of the men with a mild macrocytic anemia (mean corpuscular volume, 103; volume of packed red blood cells, 30.6) presented an extensive purpura. The platelet count was reduced to 25,000, the bleeding time was markedly prolonged, and the reticulocytes were increased. He gave a history of blood loss.

In seventeen cases (23 per cent) the anemia was normocytic. Three cases of microcytic anemia (4 per cent) were found. There was a history of definite blood loss in two of these. In the third no history of blood loss was elicited, but the stools were infested with hookworms. In all three cases both

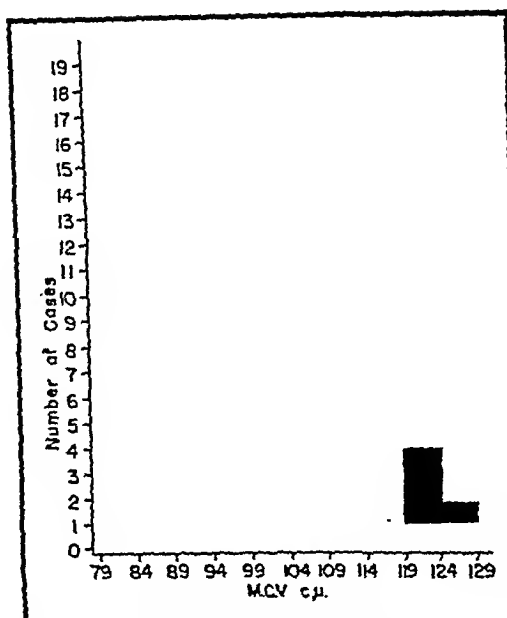


Fig. 4.—The distribution of the mean corpuscular volumes in the seventy-five subjects.

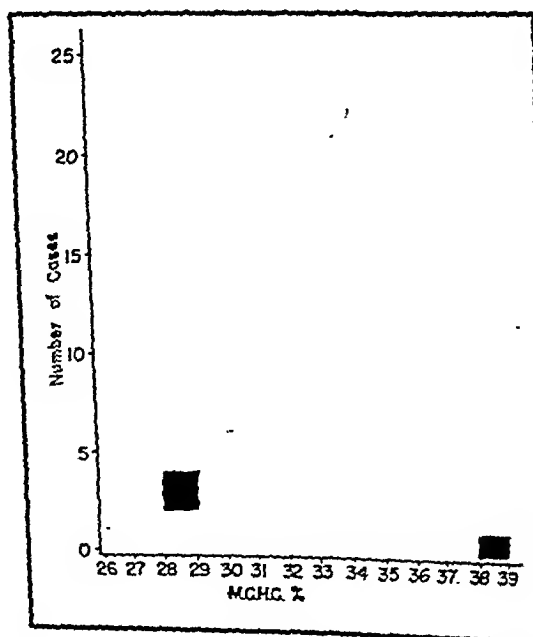


Fig. 5.—The distribution of the mean corpuscular hemoglobin concentrations in the seventy-five subjects.

the microcytosis and the hypochromia were mild. The anemia in all three instances was only mild or moderate in severity. One of these men had previously received iron.

Description of the Blood Films.—In the macrocytic group the cells appeared large and well filled with hemoglobin. A mild hypochromia was observed in a few cases only. Macrocytes were common, in some films numerous. Accompanying the macrocytosis was slight anisocytosis and moderate polychromatophilia. In a few cases the polychromatophilia was extremely marked. Nucleated red cells were occasionally seen, especially in the earlier cases studied and in those with marked macrocytosis. Stippling was seen in only one film

TABLE II. CLASSIFICATION OF ANEMIA ACCORDING TO MEAN CORPUSCULAR VOLUME

TYPE OF ANEMIA	NUMBER OF CASES	PER CENT
Macrocytic	55	73
Normocytic	17	23
Microcytic	3	4
Total	75	100

In the normocytic group the foregoing changes were seen but were less frequent. Numerous macrocytes were observed in three films, and an occasional macrocyte was found in three additional cases. In these instances anisocytosis and occasionally poikilocytosis were present. Three of the subjects presented polychromatophilia. Hypochromia was noted in three films.

Reticulocytes.—Reticulocyte counts were done in twenty of the subjects. In ten of these the percentage of reticulocytes was between 0.4 and 2.0. In the remaining ten the reticulocyte count was between 2.0 and 6.0 per cent. In only three of these subjects was the percentage 4.0 or above.

Icterus Index.—The icterus index was within the normal range in all except one case. This exception was the man with thrombocytopenic purpura.

Leucocytes.—White blood cell counts were done on fifty-three of the subjects. In nineteen the white cell count was between 5 and 10 thousand; in thirty-one the count ranged between 10 and 20 thousand (Fig. 6). In three individuals a leucocytosis greater than 20,000 was noted. In each of these a marked eosinophilia was present. No instances of leucopenia were observed.

Differential counts were not remarkable except for frequent and marked eosinophilia (Fig. 7) and an occasional mild increase in the lymphocytes or monocytes at the expense of the neutrophils. Neutropenia was not observed. Abnormal or young white cells were not seen. Multinucleated neutrophils were found in only two films.

Serum Iron.—Serum iron determinations were made on thirty-eight individuals. In seventeen of these (45 per cent) the value fell within the normal range (from 90 to 150 micrograms per cent) as determined by us in normal healthy young men. In twenty-one (55 per cent) the serum iron fell below the normal range. In seven cases the hypoferrremia was extremely severe

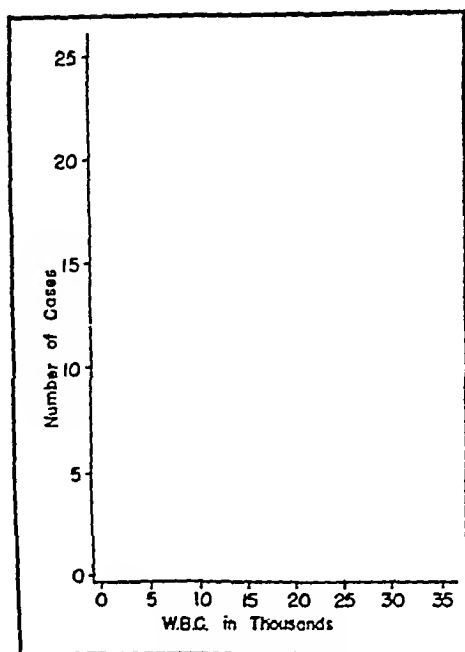


Fig. 6.—The distribution of the white cell counts in the seventy-five subjects.

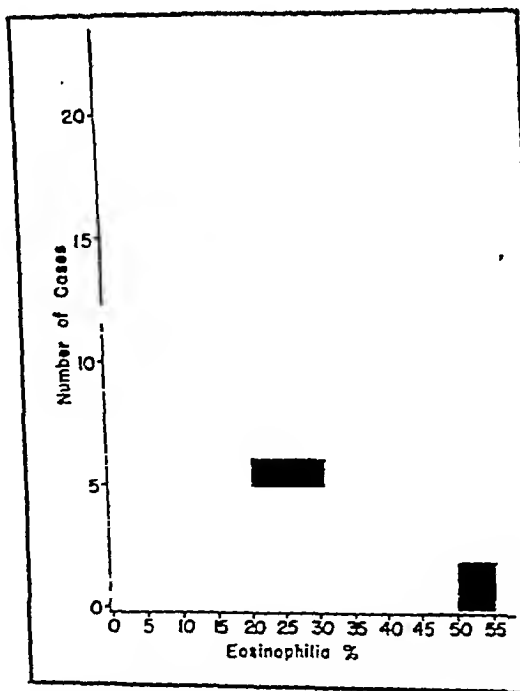


Fig. 7.—The degree of eosinophilia in the seventy-five subjects.

TABLE III. SUMMARY OF SERUM IRON AND COPPER DETERMINATIONS

CONDITION	NUMBER OF CASES	PER CENT
Normal serum iron	17	45
Hypoferremia	21	55
Hyperferremia	0	0
Normal serum copper	19	50
Hypocupremia	0	0
Hypercupremia	19	50

(below 50 micrograms per cent). The lowest value obtained was 16 micrograms per cent. The results are summarized in Table III. No case of hyperferremia was found.

Serum iron determinations done on five subjects with a mean corpuscular hemoglobin concentration of less than 30 per cent revealed a marked hypoferremia in each case.

Serum Copper.—Serum copper determinations were made on thirty-eight individuals. In nineteen of these (50 per cent) the values fell within the normal range (92 to 140 micrograms per cent). In the others the values were elevated. The highest value obtained was 215 micrograms per cent. The results are summarized in Table III. No case of hypocupremia was found.

DISCUSSION

Although the anemias seen were usually mild or moderate in degree, there is reason to believe that more severe anemia had been present at one time. During each succeeding week of the survey the incidence of anemia diminished, indicating progressive recovery. All of the men had received a wholesome diet fortified with vitamins for a period of from four to ten weeks prior to this study. Verbal reports from medical officers who examined the soldiers immediately following liberation indicate that in most cases the hemoglobin was below 50 per cent of normal at that time. It must be kept in mind that many individuals did not survive and that the most severely ill ones were not evacuated and consequently were not included in this study.

Most of the individuals were able to give a very concise and definite history. In most cases the history was probably quite reliable. These men had had a very intimate and extensive experience with the various signs and symptoms of nutritional deficiency.

Normal serum iron and copper values were found in about half of the individuals studied. Hypoferremia and hypercupremia were found in the other half. Hypoferremia is usually a manifestation of iron deficiency or infection. In general, there is a reciprocal relationship between iron and copper in the serum; that is, when the serum iron is low, the serum copper is elevated. This relationship has held in these studies. However, in experimental iron plus copper deficiency in animals, there is a depression of both the serum iron and the serum copper values. Thus in the present study no evidence of copper deficiency was found. Such a condition (hypocupremia) has never been demonstrated in human beings.

The hypoferremia accompanied by hypercupremia, together with the finding of a slight hypochromia in one-third of the subjects, is suggestive of a mild iron deficiency. It is to be noted, however, that similar changes in the iron and copper content of the serum are found in cases of anemia due to infection.³ There was no correlation between the degree of hypoferremia or hypercupremia and the degree of anemia.

This study sheds no light on the etiology of the anemia. Under the existing conditions it was not possible to study its pathogenesis. The response to specific factors could not be tested. Therefore, no conclusions can be drawn, and a discussion of the etiology must be speculative. Certain general statements can be made, however. The possible etiologic factors were blood loss, malaria, infections, parasitic infestation, and nutritional deficiency.

It is unlikely that blood loss was a factor in most of the cases. A history of blood loss was obtained in only seven patients. In six of these the blood loss was due to hemorrhoids and was extremely mild. Only one of these men gave a history of recent blood loss. Furthermore, in a great majority of cases, the anemia was macrocytic or normocytic. In the three cases of microcytic anemia it is likely that the anemia was due to blood loss in addition to an inadequate intake of iron.

Although 76 per cent of the group studied gave a history of malaria, in none was the infection recent. Malaria parasites were not found in the blood of any of our subjects. Splenomegaly was not found in any of the men, nor was the icterus index elevated. Thus, it is very unlikely that the anemia was due to malaria.

Infections, local or systemic, were not demonstrated in any of the individuals studied, excepting the one with pulmonary tuberculosis. At the time seen all individuals were afebrile and ambulatory. Although a few gave a history of pneumonia, none had had pneumonia recently. No adequate explanation for the leucocytosis is available. Whether the parasitic infestation was the cause cannot be stated. There was no correlation between the degree of leucocytosis and the degree of anemia. In general, there was no correlation between the degree of eosinophilia and the degree of leucocytosis, although in three individuals with a leucocytosis greater than 20,000 a marked eosinophilia was present (52, 55, and 21 per cent. respectively).

Intestinal parasites were demonstrated in about 75 per cent of the individuals studied. What part parasitic infestation played in the production of the anemia cannot be stated. In 25 per cent of the anemic individuals no parasites were demonstrated. Hookworm infestation was present in 32 per cent. If this parasite was the cause of the anemia, other factors probably were also concerned, since in most cases the anemia was macrocytic. Hookworm anemia is generally hypochromic and microcytic in type. As stated previously, there was no correlation between the degree of eosinophilia and the degree of anemia.

Since all but one patient had received an exceedingly inadequate diet for from thirty-nine to forty-six months and many at one time presented evi-

dence of beriberi and pellagra, it is very possible, even likely, that a deficiency of dietary factors essential for erythropoiesis existed. In some cases (about 50 per cent) this was probably complicated by malabsorption as a result of diarrhea. Since most of the individuals presented a macrocytic anemia, the anemia resembles the so-called nutritional macrocytic anemia so frequently encountered in the Orient in people who are known to subsist on a diet similar to the one on which the American prisoners of war had been placed.⁶⁻¹² One difference is apparent. Leucopenia has been frequently observed in nutritional macrocytic anemia. This was not observed in the individuals in this study. Serum iron has been reported to be normal or low in nutritional macrocytic anemia, as many cases are complicated by a mild dietary deficiency of iron.¹³ It has been demonstrated that nutritional macrocytic anemia responds to a factor, or factors, present in marmite, an autolyzed yeast preparation, and campolon, a crude liver extract.^{6, 7} Whether or not a response to purified liver extract (amaheemin) is obtainable is in dispute.¹² Wills and Evans⁶ in a series of studies concluded that the active substance is separate and distinct from the extrinsic factor of Castle. Moore and his co-workers¹³ have found that the nutritional macrocytic anemia occurring in nutritionally deficient individuals in this country responds to substances containing Castle's extrinsic factor.

SUMMARY AND CONCLUSIONS

A hematologic survey was made on seventy-five repatriated American prisoners of war selected from a series of 3,000. These individuals had been on a grossly inadequate diet consisting mainly of rice for an average of 40.1 months. The average weight loss per individual was 37 pounds. A history of nutritional deficiency as characterized by cheilosis, glossitis, stomatitis, dermatitis, and diarrhea was obtained in from 50 to 70 per cent. A history suggesting wet beriberi was obtained in 77 per cent and a history of dry beriberi in about 50 per cent.

Anemia was observed in 52 per cent of the first 1,500 men examined. The incidence of anemia diminished appreciably as successive groups were studied. Six weeks after the study was commenced, anemia was found in only 35 per cent. In most cases the anemia was only mild or moderate in degree.

Among the seventy-five anemic individuals studied in detail, the anemia was macrocytic in 73 per cent, normocytic in 23 per cent, and microcytic in 4 per cent. Thrombocytopenia was present in one patient. In this individual there was a mild elevation of the icterus index. In the remaining cases the icterus index was normal. A mild reticulocytosis and a leucocytosis (sometimes marked) were frequently encountered. Differential counts were not remarkable, except for the frequent occurrence of an eosinophilia and an occasional increase in lymphocytes or monocytes. Blood films showed numerous macrocytes, anisocytosis, polychromatophilia, and an occasional nucleated red blood cell. In approximately half of the individuals studied normal

serum iron and copper values were found. The other half were characterized by hypoferremia and hypercupremia.

The etiologic factors concerned in the production of these anemias were not studied. It is suggested, however, that most of the men examined probably presented the recovery phase of severe nutritional macrocytic anemia. The role played by intestinal parasitic infestations in the etiology of the anemia could not be determined. Malaria, blood loss, and infections were probably not important contributing factors in most of the cases. Iron deficiency may have been a contributing factor in a few. The three cases of microcytic anemia were most likely due to blood loss.

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ABSORPTION AND EXCRETION OF SULFATHIADIAZOLE AND SULFAMETHYLTHIADIAZOLE AFTER ORAL ADMINISTRATION IN HUMAN BEINGS

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THERE is still some interest in the development of nontoxic chemotherapeutic agents which are effective in the treatment of intestinal and urinary tract infections. White and co-workers¹ have recently reported a study of the antibacterial activity of some sulfacarboxythiazoles and of sulfathiadiazole against the dysentery group of organisms. The results which they obtained with two of these compounds, sulfacarboxythiazole (2-sulfanilamido-5-carboxythiazole) and sulfathiadiazole (2-sulfanilamido-1,3,4-thiadiazole), were of interest and seemed to warrant further study.

Sulfacarboxythiazole was found to be about as active as sulfaguanidine against twelve dysentery strains *in vitro* and against coliform organisms in mice. It is highly soluble at the acid pH's which may be expected in the intestinal tract, is very poorly absorbed, and presumably owes its activity to release of sulfathiazole. It is similar, in these respects, to phthalylsulfathiazole (sulfathalidine). In a limited study of sulfacarboxythiazole in human beings,² it was found to be very poorly absorbed after oral administration, produced no toxic symptoms, and appeared to be effective in a few cases of dysentery. This compound was also studied by Poth and Ross³ who found it and some related compounds to be of relatively low toxicity and high activity in the intestinal tract of the dog.

The synthesis and physical properties of sulfathiadiazole have been reported from two separate laboratories.^{4, 5} Its activity *in vitro* was found to be similar to that of sulfanilamide,^{5, 6} and in mice it was active against pneumococcal but not against streptococcal infections.⁴ Frisk⁶ also studied the pharmacology and biologic activity of the 5-methyl and 5-ethyl derivatives of sulfathiadiazole and found them to be rapidly and almost completely absorbed, nontoxic in therapeutic doses, highly soluble, rapidly and completely excreted in the urine, minimally acetylated, and biologically about as active as sulfathiazole against pneumococcus types 1 and 3 and *Escherichia coli* *in vitro* and against type 1 pneumococcal infection in mice. Frisk's monograph did not include studies on the absorption and excretion or on the *in vivo* activity of sulfathiadiazole, presumably because of the relatively low activity which he found *in vitro*.

From the comparative studies of White and co-workers,¹ however, sulfathiadiazole appeared to possess interesting possibilities for use against dysentery. As compared with sulfaguanidine it was ten times as soluble at pH 6.5, from four to eight times as active against several dysentery strains *in vitro*, and more

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active against coliform bacteria in mice. This compound did not appear to owe its activity to a decomposition product as did sulfacarboxythiazole or sulfathalidine. In preliminary pharmacologic studies, Robinson¹ found high concentrations in the intestinal contents, moderately low concentrations in the blood, and relatively high, but due to its solubility probably safe, concentrations in the urine. Studies of the absorption and excretion of this compound after oral administration in human beings, therefore, seemed warranted. Such studies are reported in this paper together with some comparative observations on the 5-methyl derivative.

MATERIALS AND METHODS

The subjects were normal laboratory workers or convalescent men patients who had not received sulfonamide drugs recently and had no evidence of cardiac or renal insufficiency. They all weighed between 66 and 77 kilograms. The first part of the study was carried out on two subjects and was essentially a repetition with sulfathiadiazole of the observations previously reported with sulfacarboxythiazole.² In the second part, a single 5 Gm. dose of sulfathiadiazole was given to each of five subjects, and the 5-methyl derivative³ was given to three subjects. Except when otherwise noted, the daily fluid intake was about 2,500 to 3,000 c.c., and no alkalis were given. All of the single doses were given at 9:00 A.M. All of the urine was collected at two-hour intervals during the first twelve hours and at twelve-hour intervals during the next thirty-six hours. Bloods for levels were obtained at these times and also one-half and one hour after the dose, but the fecal excretion was not studied. The urines passed at odd intervals also were studied in several other subjects while receiving repeated one Gm. doses after an initial four Gm. dose.

RESULTS

Excretion of Sulfathiadiazole After a Single 5 Gm. Oral Dose.—The free and total sulfathiadiazole content of the urines collected in twelve-hour periods and of each of the stools passed by two subjects are given in Table I. Subject G. B., who received no laxatives, excreted 75 per cent of the dose in the urine and 4.5 per cent in the stool. Subject B. S. was given a laxative (cascara sagrada) each morning and evening from the day before the study was begun until it was ended. A total of 60.1 per cent of the dose was recovered from the urine and 8.1 per cent from the feces of subject B. S. Of the total drug recovered from the urines, 83 per cent was excreted in the first twelve hours by G. B. and 93 per cent by B. S. In both subjects only a small percentage of the urinary sulfathiadiazole and a somewhat greater proportion of the fecal drug were in a conjugated form. The significance of the latter is not entirely clear. The blood levels at one and one-half hours were about the same in both subjects. Some drug was still present in the blood of subject G. B. eight hours after the dose was given, but none was demonstrated at that time in the blood of subject B. S.

¹The sulfonamides used in these studies were furnished by the Lederle Laboratories, Inc., Pearl River, N. Y. The chemical determinations were carried out by Ellen J. Doyle.

Excretion of Sulfathiadiazole After Repeated Oral Doses.—The same two subjects were later given repeated doses of sulfathiadiazole, and the amounts recovered from urine and feces were determined. In addition, blood levels were determined hourly for four hours after the initial 4 Gm. dose and twice thereafter. The results are shown in Table II. In this instance the total amounts of drug recovered in the urine and feces were quite comparable in the two subjects, most of it being found in the urine. More of the urinary sulfathiadiazole in subject B. S. and less of the fecal drug recovered in both subjects were in the conjugated form. The maximum blood level was obtained two hours after the initial 4 Gm. dose in G. B. and one hour after that dose in B. S., but in both subjects appreciably lower levels were found in the thirty-two and forty-nine hour bloods which were drawn four hours and one hour, respectively, after a 1 Gm. dose.

The concentration of the drug in the different specimens varied depending, of course, on the volume of urine excreted. In Subject G. B. the concentrations ranged from 540 to 240 mg. per cent for the total and from 500 to 182 mg. per cent for the free drug during the time when it was being given. In subject B. S. the range was between 430 and 160 mg. per cent for the total and between 410 and 100 mg. per cent for the free drug. Neither crystals nor red blood cells were found in any of the specimens, but the subjects took fluids liberally (about 3 liters per day).

TABLE III. SULFATHIADIAZOLE IN INDIVIDUAL SPECIMENS OF URINE DURING ADMINISTRATION OF REPEATED ORAL DOSES

SUBJECT	DAY	ESTIMATED FLUID INTAKE (C.C. PER DAY)	PH OF URINE	DRUG IN DIFFERENT SPECIMENS OF VOIDED URINE (MG. %)				PER CENT CONJUGATED	
				MAXIMUM		MINIMUM		RANGE	AVERAGE
				FREE	TOTAL	FREE	TOTAL		
J. S.	1	1,500	5.8 to 7.0	1,030	1,180	610	670	9 to 12	10.8
	2	2,500	5.4 to 5.7	900	910	170	190	4 to 27	10.4
	3 to 4	3,500*	7.2 to 8.8	410	450	70	95	4 to 24	16.5†
L. G.	1	5,000	6.5 to 7.0	415	480	10	10	0 to 14	11.6
	2	900	5.2 to 6.0	500	584	150	190	4 to 24	11.3
	3	2,500	5.2 to 6.5	390	450	240	310	13 to 22	18.5‡
R. L.	1 to 4	3,500	5.3 to 6.8	202	232	64	70	9 to 21	14.2

*Sodium bicarbonate, 6 Gm. followed by 2 Gm., with each dose given on these days.

†Average for the four days was 11.6 per cent.

‡Average for the three days was 13.3 per cent.

Three additional patients were given 4 Gm. each of sulfathiadiazole followed by 1 Gm. every four hours, and all voided specimens were collected during the time when the drug was being administered. The pH of each specimen was tested, and the concentrations of free and total sulfathiadiazole were determined. The results are summarized in Table III in relation to the fluid intake. The expected effect of the fluid intake is clearly shown. Several specimens from Subject J. S. contained more than 1 per cent of drug. The sediments of all the voided specimens in these cases were examined and found to be free of red blood cells and drug crystals.

Rapidity of Absorption and Excretion of Sulfathiadiazole and Sulfamethylthiadiazole After a Single Oral Dose.—Since most of the drug which appeared in the urine after a single 5 Gm. oral dose of sulfathiadiazole was recovered during the first twelve hours, it was of interest to determine in more detail when the maximum absorption and excretion occurred. For that purpose a single 5 Gm. dose was given to five subjects, and the total urine output was collected at two-hour intervals during the first twelve hours and at twelve-hour intervals for the next thirty-six hours. Blood levels were determined at these intervals and also at one-half and one hour. A similar study was carried out with sulfamethylthiadiazole in three subjects, two of whom were also used for the sulfathiadiazole study. The results are shown graphically in Figs. 1* and 2.

For both drugs the rapidity of absorption varied considerably as indicated by the maximum blood levels and the time when they were attained. The maximum concentrations of total sulfathiadiazole in the blood varied from 4.0 to 7.8 mg. per cent and the free drug varied from 3.6 to 6.7 mg. per cent. The corresponding figures for sulfamethylthiadiazole were from 6.5 to 12.0 mg. per cent total and from 6.2 to 10.8 mg. per cent free. These maximums were attained in different subjects from one to four hours after the ingestion of either drug.

TABLE IV. URINARY EXCRETION OF SULFATHIA DIAZOLE AND SULFAMETHYLTHIA DIAZOLE AFTER SINGLE 5 GM. DOSE

SUBJECT	PERCENTAGE OF TOTAL DRUG EXCRETED IN THE URINE RECOVERED IN THE FIRST				
	2 HOURS	4 HOURS	6 HOURS	10 HOURS	24 HOURS
<i>Sulfathiadiazole</i>					
J. M.	3	28	58	88	100
E. O.	5	45	69	93	99
R. L.	21	39	70	90	98
M. M.	22	65	89	97	99
B. S.	44	83	91	96	100
<i>Sulfamethylthiadiazole</i>					
E. O.	8	29	52	90	99
J. B.	5	47	73	95	99
M. M.	50	83	95	99	100

The urinary excretions showed corresponding differences. The maximum excretion occurred in different subjects, either in the first, second, or third two-hour interval. The greatest variations in excretion occurred in the first and second two-hour periods. Practically all of the urinary excretion occurred in the first twenty-four hours, and very little was excreted after ten hours in most of the subjects and after six hours in some of them. This is shown in Table IV. The total amount of drug excreted by each of the subjects and the proportion which was determined as being in the conjugated form are shown in Table V. From 60 to 100 per cent of the administered sulfathiadiazole (average, 83.4 per cent) was recovered in the urine, while the sulfamethylthiadiazole was completely recovered in the urine in each of the three subjects.

The maximum concentrations found in the urine in these subjects are shown, together with other relevant data in Table VI. The maximum concentration of

*The findings in subject J. M. are omitted from this figure because frequent blood levels were not determined.

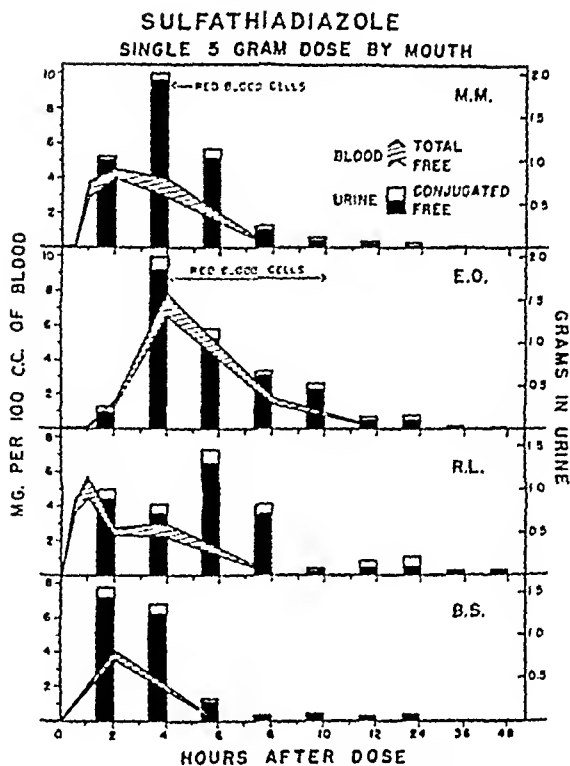


Fig. 1.—Blood levels and urinary excretion of sulfathiadiazole in four subjects each given a single dose of 5 Gm. by mouth.

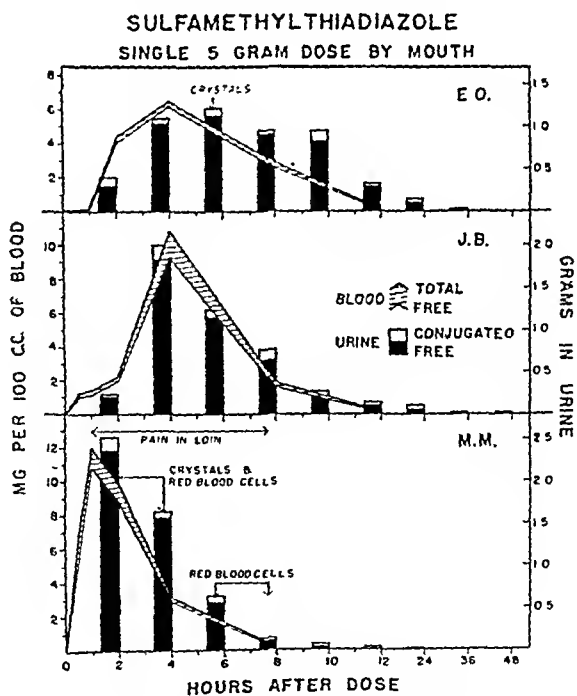


Fig. 2.—Blood levels and urinary excretion of sulfamethylthiadiazole in three subjects each given a single dose of 5 Gm. by mouth.

TABLE V. TOTAL URINARY EXCRETION OF SULFATHIAZOLE AND SULFAMETHYLTHIAZOLE AFTER SINGLE ORAL 5 GM. DOSE

SUBJECT	GM. IN URINE		PER CENT OF DOSE EXCRETED	PER CENT CONJUGATED
	FREE	TOTAL		
Sulfathiadiazole				
B. S.	2.687	3.003	60.1	10.5
B. S.	3.172	3.511	70.2	9.6
G. B.	3.335	3.749	75.0	11.0
J. M.	4.142	4.541	90.8	8.8
R. L.	3.955	4.700	94.0	15.8
M. M.	4.392	4.702	94.0	6.6
E. O.	4.515	4.987	99.7	9.5
Average	3.743	4.170	83.4	10.2
Sulfamethylthiadiazole				
J. B.	4.239	4.805	96.1	11.8
M. M.	4.737	5.046	100.9	6.2
E. O.	4.801	5.264	105.3	8.8
Average	4.592	5.038	100.8	8.9

TABLE VI. MAXIMUM CONCENTRATION OF SULFONAMIDES IN URINE AFTER SINGLE 5 GM. DOSE

SUBJECT	INTERVAL AFTER DOSE (HR.)	VOLUME (C.C.)	PH	MG. PER 100 C.C.		SEDIMENT	
				FREE	TOTAL	CRYSTALS	R.B.C.
Sulfathiadiazole							
J. M.	2 to 4	385	--	293	300	0	0
E. O.	2 to 4	125	5.5	1,480	1,600	0	Few*
R. L.	2 to 4	65	5.5	1,100	1,270	0	0
M. M.	2 to 4	180	6.0	1,320	1,380	0	Rare
B. S.	2 to 4	290	--	428	472	0	0
Sulfamethylthiadiazole							
E. O.	4 to 6	90	5.8	1,260	1,360	Many	0
J. B.	2 to 4	150	5.5	1,220	1,360	0	0
M. M.	0 to 2	175	6.0	1,360	1,440	Many	Many†

*There were occasional red blood cells but no crystals in the sediment of later specimens collected up to eight hours.

†Two hours after the dose this subject developed pain in the right loin radiating to the groin and accompanied by nausea. Crystalluria was also present in the two- to four-hour specimen, and microscopic hematuria persisted for four hours longer. The two- to four-hour specimen consisted of 150 c.c., pH 5.5, and contained 1,060 mg. per cent free and 1,100 mg. per cent total drug.

sulfathiazole was found in the two- to four-hour specimen in each instance and varied from 300 to 1,600 mg. per cent. The concentration of the methyl compound reached from 1,360 to 1,440 mg. per cent in the three subjects, but these maximums were found at different intervals after the dose. Only a small percentage of the drug was found in the urine in the conjugated form. These specimens were all acid (pH 5.5 to 6.0).

Distribution Between Plasma and Blood Cells.—The distribution of sulfathiazole and sulfamethylthiazole between plasma and red blood cells was studied in two subjects at the time of the maximum blood level after a single oral dose. The results are shown in Table VII. The plasmas contained 2.5 and 3.1 times as much free sulfathiazole and fifteen and twenty-four times as much free sulfamethylthiazole as the red blood corpuscles. All of the conjugated drug was in the plasma, and none was found in the red blood cells.

Untoward Effects.—One patient, subject J. B., developed fever (100° F.), conjunctivitis, and urticaria after forty-eight hours of treatment with sulfamethylthiazole given in the usual dosage, namely, 4 Gm. followed by 1 Gm.

TABLE VII. DISTRIBUTION IN PLASMA AND RED BLOOD CELLS

SUBJECT	SULFATHIAZOLE		SULFAMETHYLTHIAZOLE	
	E. O.	M. M.	E. O.	M. M.
Whole blood (mg. %)				
Free	6.6	4.0	6.2	10.8
Conjugated	1.2	0.5	0.3	1.2
Plasma (mg. %)				
Free	8.9	5.8	10.4	17.0
Conjugated	2.2	1.3	1.7	2.8
Hematocrit (%)	43	45	43	45
Red blood cells (mg. %) (calculated)				
Free	3.5	1.9	0.7	0.7
Conjugated	0	0	0	0

every four hours. He had received a single 5 Gm. dose four days previously without untoward effect. Fever and symptoms subsided within a few hours after the last dose. The patient was subsequently given test doses of 2 Gm. of sulfathiazole and sulfadiazine and showed no untoward effects. The only other untoward effects observed are those noted in Table VI. It is of interest, however, that several other subjects had concentrations of more than 1 per cent of sulfathiazole in urine that was acid at the time and nevertheless showed no hematuria or crystalluria. There was no nausea noted except during the episode of renal colic in Subject M. M. The sulfamethylthiazole crystals recovered from the urine of subjects E. O. and M. M. were probably in the free form as judged by the usual chemical determination. They appeared microscopically as large compact sheaths with irregular and jagged ends resembling broken-off tree trunks.

DISCUSSION

The types of compounds which are considered to be useful in the treatment of enteric infections are those which are highly soluble at the acid pH of the intestinal contents and poorly absorbed, so that high concentrations are maintained in the bowel and very little gets into the blood and urine. If there is good absorption, the excreted drug must be highly soluble at the range of pH that may be expected in the urine. For the treatment of urinary tract infections it is desirable to have compounds which are absorbed and excreted fairly rapidly with a minimum of acetylation and are highly soluble in the range of pH of the urine. Both types of drugs must be active, of course, in their respective media against the pathogenic organisms for which they are used.

Sulfathiazole and sulfamethylthiazole were both found to be rapidly absorbed and excreted minimally conjugated in blood and urine after oral administration. All but a small percentage of the sulfathiazole and practically all of the 5-methyl derivative were recovered from the urine, largely in the free form and mostly within the first few hours after they were ingested. Only from 4.5 to 8.1 per cent of the orally administered sulfathiazole were recovered from the feces. High concentrations, often over 1 per cent, were obtained in the urine in the first few hours after a single 5 Gm. dose. In most instances these large amounts of drug remained in solution in the urine, even at pH as low as 5.5. In two subjects, however, sulfamethylthiazole crystals appeared

in large numbers in the voided urine after a single 5 Gm. dose, and this was accompanied by renal colic and hematuria in one of them. A few red blood cells without crystals were also present in the urine in two subjects after a dose of sulfathiadiazole, but in every instance the urine contained very high concentrations of drug at the time. These complications occurred within a few hours after a 5 Gm. dose and were not seen during repeated administration of 1 Gm. doses at four-hour intervals.

In the preliminary studies of White and co-workers¹ sulfathiadiazole appeared to have therapeutic possibilities for the treatment of dysentery infections and seemed to be active against coliform organisms in mice. The present studies, however, suggest that, from the point of view of its absorption and excretion, this compound is not particularly suited for enteric infections unless the small amounts remaining in the bowel are very highly active. Such a possibility is suggested by the effectiveness of sulfathiazole and sulfadiazine in the treatment of dysentery in spite of the small amounts recoverable in the feces, and sulfathiadiazole has the added advantage of greater solubility at the pH of the intestinal contents. On the other hand, the high solubility throughout the urinary pH range and the effectiveness against coliform organisms suggest that sulfathiadiazole and its 5-methyl derivative may be useful in urinary tract infections. Doses as large as 5 Gm. at a time, however, should be avoided, but doses of 1 Gm. each can probably be given safely every four hours. More experience in the actual treatment of urinary tract infections is needed before the safety of these compounds and their effectiveness can be determined.

CONCLUSIONS

Sulfathiadiazole given by mouth was rapidly and almost completely absorbed, rapidly eliminated in the urine, and highly soluble in the urinary range of pH. About 90 per cent of the drug recovered from the urine was in the free form. Sulfamethylthiadiazole was more completely absorbed and rapidly eliminated in the urine, and it gave somewhat higher maximum blood levels. Microscopic hematuria and macroscopic crystalluria were observed in two of three subjects after a single oral dose of 5 Gm. of the methyl derivative. The findings suggest that sulfathiadiazole deserves a trial in the treatment of urinary tract infections but that large individual doses should be avoided.

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THE QUANTITATIVE DETERMINATION OF THE THYMOL TURBIDITY REACTION OF SERUM

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THE thymol turbidity reaction of serum as described by Maclagan^{1, 2} has been studied, especially in liver disease, by several observers.^{3, 4} Shank and Hoagland⁵ have adapted the reaction to the Coleman spectrophotometer, using barium sulfate suspensions as standards.

The determination of Maclagan units of turbidity by the visual comparator block is not precise enough for the statistical analysis of the results. In this paper is reported a modification of the Maclagan method which appears to offer a more quantitative approach to the determination of thymol turbidity as a means of evaluating the presence of liver disease.

METHODS

The entire reaction with serum, as well as the formation of the standard barium sulfate suspensions, was carried out in Evelyn tubes. A 660 μ filter was used in an Evelyn colorimeter. Galvanometer readings G were changed to L values ($2 - \log G$). Distilled water was used for the 100 setting on the colorimeter rather than the thymol buffer, as the latter tends to become turbid upon standing. Slight turbidity of the thymol buffer clears when it is added to the serum, but in general cloudy buffer should not be used.

Standard Barium Sulfate Curve.—A barium sulfate suspension was prepared as described by Shank and Hoagland,⁵ using 3 c.c. of 1 per cent barium chloride diluted to 100 c.c. with 0.2 N sulfuric acid. Serial dilutions of the standard were made with 0.2 N sulfuric acid, and the resulting turbidity was measured in the Evelyn photoelectric colorimeter. Seven serial dilutions of the barium sulfate suspension were made, each one five times. The mean value for each dilution was obtained and a regression equation derived from the data. The equation is: $Y = 20.81X - 0.0271$, where Y = c.c. BaSO_4 suspension, and X = L value ($2 - \log G$). The values obtained are shown in Table I. The curve drawn from these values is a straight line. The approximate Maclagan units corresponding to the dilutions of the BaSO_4 standard are given in Table I.

Procedure.—The reaction was carried out as follows: Into an Evelyn tube were placed, in succession, 0.1 c.c. of serum and 6.0 c.c. of thymol-barbiturate buffer prepared according to Maclagan.² The contents were well mixed and kept at room temperature for thirty minutes; galvanometer readings were then obtained using distilled water as the 100 setting.

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TABLE I. RELATION BETWEEN VOLUME OF BARIUM SULFATE SUSPENSION, EVELYN READINGS, AND MACLAGAN UNITS

BA ₂ SO ₄ SUSPENSION (C.C.)	0.2 N H ₂ SO ₄ (C.C.)	EVELYN GALVANOMETER READING	L VALUE (2-LOG G)	APPROXIMATE MACLAGAN UNITS
0.5	5.5	94 ²	0.0246	2 ½
1.0	5.0	89 ⁰	0.0505	5
2.0	4.0	79 ³	0.0982	10 ½
3.0	3.0	71 ²	0.1457	16
4.0	2.0	64 ⁰	0.1936	22
5.0	1.0	57 ⁰	0.2441	28
6.0	0.0	31 ¹	0.2903	34

The galvanometer readings *G* were changed to *L* values ($2 - \log G$), and the corresponding cubic centimeters of barium sulfate suspension, obtained from the equation by calculation or from the curve, were taken as indicating the degree of equivalence of thymol turbidity.

RESULTS

Normal.—Serum was obtained from 105 apparently healthy young men without a history of gastrointestinal disturbance or jaundice within three months. The serum bilirubin concentrations⁶ were normal in all.

The thymol turbidity of these men varied from 0.16 c.c. to 2.19 c.c. of barium sulfate (Table II). The mean was 0.63 c.c. with a standard deviation ($S.D. = \sqrt{\frac{\sum d^2}{n-1}}$) of 0.35 cubic centimeter. If the the mean normal plus three times the standard deviation is taken as the upper limit of normal variation, this would give normal limits of thymol turbidity as equivalent to from 0 to 1.68 c.c. of the barium sulfate suspension. In our normal group two sera had values above this.

Cirrhosis of the Liver.—Sixty-three thymol turbidity determinations were made on twenty-three individuals with cirrhosis of the liver (Laënnec). Twelve of the cases were arbitrarily classified, without reference to the thymol turbidity, as mild and eleven as severe. The results are shown in Table II. Eighty-one per cent of the determinations in all cirrhosis were elevated above 1.68 c.c., while in advanced cirrhosis 92 per cent were likewise above normal.

TABLE II. THYMOL TURBIDITY REACTION OF NORMAL SERA CONTRASTED WITH REACTION IN LAËNNEC'S CIRRHOSIS AND ILLNESSES WITHOUT EVIDENCE OF LIVER DISEASE

THYMOL TURBIDITY (C.C. BA ₂ SO ₄)	DISTRIBUTION IN PERCENTAGE				
	NORMAL	ILLNESSES WITHOUT EVIDENCE OF LIVER DISEASE	LAËNNEC'S CIRRHOSIS		
			ALL	MILD	SEVERE
Number of determinations	105	27	63	26	37
< 1	91	33	8	15	3
1.0 to 1.68	7	33	11	15	5
1.68 to 2.0	2	7	9	23	3
2.0 to 3.0	0	7	35	31	38
3.0 to 4.0	0	0	29	16	30
4.0 to 5.0	0	0	8	0	21

Liver Disease Other Than Laënnec's Cirrhosis.—In Table III are shown the thymol turbidities of sixteen patients with other forms of liver disease than Laënnec's cirrhosis. The highest values were found in infectious hepatitis, but values above normal were also found in the sera from patients with hemolytic anemia with liver disease, carcinoma with liver metastases, and homologous serum jaundice. High values were also found in infectious mononucleosis, but this cannot be taken alone as evidence of the presence of liver disease in this condition.

TABLE III. THYMOL TURBIDITY IN LIVER DISEASE OTHER THAN LAËNNEC'S CIRRHOSIS

DISEASE	NUMBER OF PATIENTS	THYMOL TURBIDITY (C.C. BARS. SUSPENSION)
Hemolytic anemia with liver disease	2	1.10, 2.30
Biliary cirrhosis	1	0.95
Familial nonhemolytic icterus	1	1.05
Hepatic vein obstruction	1	0.90, 1.00, 0.25
Carcinoma with liver metastases	1	0.90, 2.00, 1.75, 2.65
Infectious hepatitis	4	1.45 to 7.10
Infectious mononucleosis	2	1.50, 4.60
Homologous serum jaundice	1	1.30 to 4.85

Patients Without Evidence of Liver Disease.—The thymol turbidity was measured on the sera of twenty-seven hospitalized patients with illnesses not associated with liver disease. The results are shown in Table II where they may be compared with those obtained in Laënnec's cirrhosis. Only 14 per cent had turbidity values of 1.68 c.c. of barium sulfate or more.

COMMENT

The thymol turbidity described by Maelagan¹ has the advantage over most tests depending upon qualitative or quantitative changes in the serum proteins of being simple and rapid. In thirty-one normal individuals, Watson and associates⁴ found values of from 1 to 4 Maelagan units and Shank and Hoagland⁵ of from 0. to 4.7. The range of values in cirrhosis of the liver and infectious hepatitis is quite wide, but many are within or near the normal range. Thus, it is often difficult to decide whether a value is abnormal or not.

The modification of the method described herein makes it possible to determine more precisely the range of normal as well as the significance of abnormal values without the use of arbitrary units. Inspection of Table II shows that only 2 per cent of normal individuals had values above 1.68 c.c. of barium sulfate; however, 14 per cent of hospitalized patients without evidence of liver disease were above normal, and 30 per cent of the determinations in mild cirrhosis were within the normal range. Thus, a negative test is of no value in eliminating cirrhosis of the liver. However, a positive test equivalent to 4 c.c. of barium sulfate suspension or above is good evidence of liver disease, as none of the sera obtained in illnesses without liver disease were above this figure.

The cases of other forms of liver disease are too few for statistical analysis. However, Shank and Hoagland,⁵ in infectious hepatitis, found eighty-two of

eighty-three patients with increased thymol turbidity, and Cohn and Lidman,⁷ in fifteen soldiers with infectious mononucleosis, found a high incidence of positive reactions, as well as other evidence of liver disease.

SUMMARY

1. A modification of the Maclagan thymol turbidity reaction of serum is described, using the Evelyn photoelectric colorimeter.

2. For greater accuracy and convenience, the values for turbidity are expressed as cubic centimeters of the barium sulfate suspension used as standard. The approximate Maclagan units determined visually are presented for comparison.

3. The thymol turbidity of 105 apparently healthy young men varied from 0.16 to 2.19 c.c. of barium sulfate suspension. The upper limit of normal, using three times the standard deviation, was 1.68 cubic centimeters.

4. In cirrhosis of the liver, 81 per cent of the determinations were abnormal; in severe cirrhosis, 92 per cent. In a series of illnesses without evidence of liver disease, 14 per cent were above normal but none above 3 c.c. of barium sulfate suspension; whereas, 37 per cent of the determinations in cirrhosis were above this figure.

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LABORATORY METHODS

SEMIQUANTITATIVE ESTIMATION OF BILIRUBIN IN THE URINE BY MEANS OF THE BARIUM-STRIP MODIFICATION OF HARRISON'S TEST

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THE importance of detecting bilirubin in the urine has recently been emphasized with respect to the diagnosis of infectious hepatitis, both in the preicteric stage of the disease and in those cases in which jaundice does not become manifest.¹⁻⁴ The barium-strip modification of the Harrison test for bilirubin in the urine, as described in a previous communication,⁵ was sought and elaborated in order to provide a method which would be suitable for mass and serial usage, as in an epidemic of infectious jaundice, or in a chemical industry where injury of the liver was a possible hazard. Further experience with this technique has confirmed our belief in its simplicity and the rapidity with which it may be executed, also its specificity and sensitivity. The only other method which might be compared with it, on the basis of these attributes, is the methylene blue test, which, however, is not quite as sensitive nor as uniformly specific for bilirubin and in addition is more time consuming.

A description of the barium-strip modification of Harrison's test is given in the following:

Sheets of thick retentive filter paper* are thoroughly immersed in a saturated aqueous solution of barium chloride, after which they are dried either in a drying oven or at room temperature. The sheets are then cut into strips 3 or 4 inches by $\frac{1}{2}$ inch in dimension. These strips may be prepared in any desired number, as they keep indefinitely.

To carry out the test, one of the barium-impregnated strips is simply inserted perpendicularly into the urine sample in question. It is held at the same level for from five to ten seconds, and at least $\frac{1}{4}$ inch of the lower end of the strip should be below the surface of the urine. During this period it is seen that the urine runs up the strip, but the pigments are adsorbed and collected just at the surface. The strip is removed and placed in the horizontal position on white absorbent paper such as ordinary filter paper or paper towel. At the point where the strip was at the surface of the urine, a yellow or brown line, or zone, is seen running transversely across the strip. This is usually present, although faintly, even with most normal urines. The Fouchet reagent, as used in the Harrison test, is dropped from a dropping bottle directly on this zone which was just at the surface of the urine. A green

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*Schleicher and Schull, No. 470. Thus far, we have not had experience with any other type of paper which was satisfactory.

BARIUM-STRIP MODIFICATION OF HARRISON'S TEST
FOR BILIRUBIN IN THE URINE

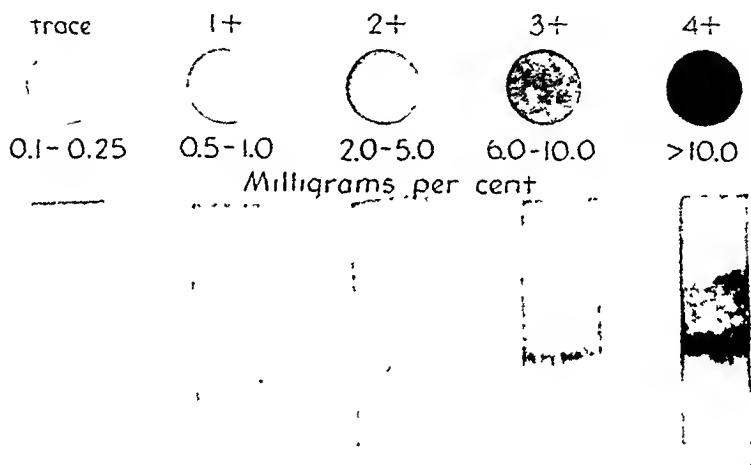


Fig. 1.

color denotes the presence of bilirubin. If just a trace is present, one may see only a very faint green line, while with larger amounts, an intense dark green is observed to spread up and down the strip over a broad area. The green color alone is specific for bilirubin. At times a purple or reddish color is seen, most likely due to dehydrogenation products of urobilinogen.

The foregoing procedure has been rendered semiquantitative by the use of a color chart of five standard intensities of green color, as shown in Fig. 1. These colors were painted* from the barium-strip Harrison tests of as many concentrations of bilirubin, each dissolved in pooled normal urine. The sample barium-strip tests, as shown in the lower part of Fig. 1, were carried out on pathologic urines containing varying amounts of bilirubin. They simply illustrate the means by which the comparisons with the standards are made.

This simple, semiquantitative method facilitates observation of the degree of bilirubinuria, whether increasing or decreasing, and thus aids in establishing the trend of the disease. Furthermore, it offers a more nearly standard means of reporting the degree of bilirubinuria which hitherto has been largely a matter of the personal equation.

SUMMARY

A standard color chart is reproduced by means of which the barium-strip modification of the Harrison test for bilirubin in the urine has been made semiquantitative in character.

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*We are indebted to Miss Jean Hirsch, University Medical Art Shop, for this work.

APPLICABILITY OF THE BIURET REACTION TO THE DETERMINATION OF SERUM ALBUMIN BY METHANOL PRECIPITATION

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PILLEMER and Hutchinson¹ have shown that the precipitation of globulins by aqueous methanol under controlled conditions yields results for serum albumin comparable to those obtained by electrophoretic measurement. The protein partition obtained with sodium sulfate according to Howe² is known to yield falsely high values for albumin.^{3, 4} In this laboratory the method of Pillemer and Hutchinson has been found convenient and useful. Further simplification has been obtained through the observation that albumins can be determined in the filtrates by the biuret reaction essentially according to Kingsley,⁵ in spite of the presence of about 40 per cent methanol. This procedure avoids the difficulties inherent in the complete recovery of protein nitrogen by micro-Kjeldahl procedures.⁶

PROCEDURE

A 1 c.c. aliquot of the methanol-albumin supernatant (separated by spinning in a small centrifuge kept in the refrigerator) is transferred to a dry cuvette. In another cuvette is placed 0.1 c.c. of the original serum (for the total protein determination) and in a third cuvette (for the reference blank), 0.1 c.c. of water. To the latter two tubes is added 0.9 c.c. of acetate-methanol mixture (made by mixing 1 volume of acetate buffer, 7 volumes of aqueous methanol, both prepared according to Pillemer and Hutchinson, and 1 volume of water). To all cuvettes are added with mixing 4.3 c.c. of 3 N sodium hydroxide and 0.7 c.c. of 1 per cent copper sulfate. After just fifteen minutes the transmittances are determined in a photoelectric colorimeter or spectrophotometer. The absorption maximum is at a wave length of about 555 millimicrons.

The colorimeter is calibrated by measuring transmittances of several dilutions of a serum whose protein content has been determined by the Kjeldahl methods. The figures read from the curve relating log transmittance to per cent protein are halved for the albumin determination. With lactescent or highly icteric sera one may add 2 c.c. of ether as suggested by Kingsley. However, the correction of + 8 per cent used by Kingsley is omitted since dilution produced by dissolution of ether is approximately compensated by extraction of methanol (Table I).

With the spectrophotometer* at wave length 555 millimicrons, a linear relationship was obtained between log transmittance and concentration, the color being about 6 per cent more dense than that obtained in the absence of methanol.

From The Mary Imogene Bassett Hospital.

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*Model 11, Coleman Electric Co.

TABLE I. EFFECT OF ETHER ADDITION UPON TRANSMITTANCE OBTAINED WITH SERUM

SERUM	PER CENT TRANSMITTANCE	
	ETHER NOT ADDED	ETHER ADDED
1	31.3	31.3
2	36.3	35.9
3	30.8	29.9
4	26.5	26.0

TABLE II. COMPARISON OF ALBUMIN FOUND IN METHANOL FILTRATES BY BIURET REACTION AND BY KJELDAHL TECHNIQUE

SERUM	PER CENT SERUM ALBUMINS FOUND	
	BY KJELDAHL	BY BIURET REACTION
5	2.87	2.8
6	3.34	3.3
7	2.42	2.3
8	3.69	3.8

The presence of methanol apparently did not reduce the accuracy of the biuret method (Table II)..

SUMMARY

The biuret method of Kingsley has been adapted to the measurement of serum albumin in filtrates obtained by methanol by the method of Pillemer and Hutchinson.

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OXYGEN CONSUMPTION AND DRUG ACTION: A METHOD FOR MEASUREMENT OF THE RESPIRATION OF AQUATIC ANIMALS

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MANY drugs affect tissue respiration, and in order to study the relationship between drug action and cellular metabolism it is frequently necessary to make measurements of the rate of oxygen consumption. With isolated cells or tissue slices the Warburg manometric technique is applicable; however, with larger intact organisms it is impractical to maintain oxygen equilibrium by shaking the immersion fluid, and some other method must be used. A modification of the constant-flow manometric respirometer, described previously for use with small mammals,¹ has been shown to be suitable for this purpose. The method is simple and determinations may be made at frequent intervals. This article describes the technique and presents illustrative results obtained with the frog and the quahog, *Venus mercenaria*, using hydrocyanic acid as the experimental drug.

Fig. 1 is a photograph of the equipment in operation. It consists essentially of three plastic chambers immersed in a constant-temperature water bath. Two of these are connected with a pump that continuously circulates air through them, and the third chamber is attached to a Warburg manometer and acts as a thermobarometer. The chambers are made of $4\frac{3}{4}$ inch Lucite tubing with $\frac{1}{8}$ inch walls, closed at the bottom with a plate of plastic. They fasten tightly to the $\frac{3}{8}$ inch top plate by means of a simple inclined plane-locking device. This connection is sealed with a lanolin-petrolatum-mineral oil mixture each time the chambers are closed.

Air from the pump bubbles through the water in the first chamber, which contains the experimental animal, and then passes through a connecting glass tube to the second chamber. Carbon dioxide is removed from here by a tube of 5 per cent potassium hydroxide solution and an alkali-soaked gauze. The air is then led back to the pump through a thick-walled capillary tube and repeats the circuit. A lead from the top of the second chamber goes to a Warburg manometer, and any change in pressure within the system is thus observable. Since the carbon dioxide is absorbed and the temperature remains constant, the pressure changes (after correction for barometric changes recorded by the thermobarometer) are due to oxygen consumption by the animal in the water.

Air is circulated through the system by a rubber-tube pump which delivers about 250 c.c. a minute. A $\frac{3}{8}$ by 22 inch piece of soft gum rubber tubing is coiled once inside a cylindric hole in a wooden block, with each end passing through the block and attaching to glass connecting tubes. The air is continuously driven through the rubber tubing by a free-moving wooden roller

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which pushes the air ahead of it as it squeezes the rubber. This roller is driven by friction contact with another roller of about the same diameter, which is connected to the driving motor with a rubber coupling. The driving motor is one that is used for a Warburg manometer shaker and has a gear reduction which gives a speed of 120 revolutions per minute.

When the closed arm of the manometer is reset to the starting level before each reading, the system constitutes a constant volume type of respirometer, and the theory is similar to that of the direct method of Warburg.² If the volume and the temperature are kept constant, it is only necessary to multiply the thermobarometer-corrected manometer reading by a constant to determine

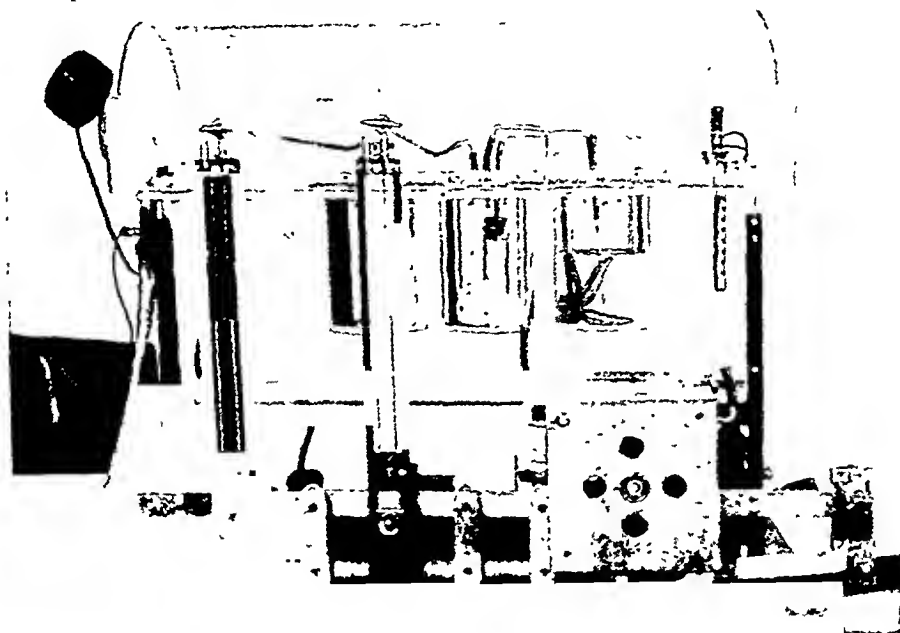


Fig. 1.—Photograph of closed-circuit manometric respirometer for measuring the oxygen consumption of aquatic organisms.

the amount of oxygen consumed, corrected to standard temperature and pressure conditions. This constant depends primarily upon the gas volume of the system, and the sensitivity may be varied over a wide range by changing the size of the chambers or the amount of fluid they contain. The total volume may be determined easily by measuring the amount of water the system will hold.

DETAILS OF TECHNIQUE

Oxygen equilibrium between the water and the air in the system is attained rapidly when the stream of flowing air is sufficient, and in most cases the lag in manometric response to a change in the respiration of the animal is insignificant. The water in the chambers should be allowed to come to the temperature of the thermoregulated bath before the initial readings are taken.

To maintain an equivalent vapor tension, the thermobarometer chamber should contain a small quantity of water.

Addition of oxygen to replace that which has been used by the animal is made by means of a rubber anesthesia bag filled with the gas and attached to the top opening of the manometer. The system is brought back to the original oxygen tension at any time by simply turning the stopcock of the manometer and allowing the gas to flow in.

Removing the carbon dioxide from sea water leads to an increase in alkalinity,³ and since the potassium hydroxide in the second chamber combines with this gas, a change in pH may occur if marine animals are being studied. A buffer may be added,⁴ but ordinarily this is unnecessary, since the carbon dioxide production of the respiring material is usually sufficient to maintain equilibrium. When the volume of the liquid is relatively large, the change in pH is insignificant for experiments of a few hours' duration, even if there is little carbon dioxide production.

Several connections are made with rubber tubing, and it is advisable to test the equipment occasionally for leaks. This may be done by setting it up without respiring material and comparing the readings with those of the thermobarometer. If the pump is well made and a good quality of gum rubber is used, the pump tubing will run for months before it requires replacement.

The apparatus is particularly well suited for observations of the effect of cyanide on oxygen consumption. This may be done by inserting a bubbler of calcium cyanide-calcium hydroxide mixture in the second chamber to add hydrogen cyanide gas to the air after the carbon dioxide has been removed. It has been shown that the hydrogen cyanide tension of such a mixture varies with the concentration of the calcium cyanide and with the temperature,⁵ and it is thus possible, by use of the proper solution, to maintain a constant concentration of cyanide in the animal chamber and still remove the carbon dioxide

TABLE I. CALCIUM CYANIDE SOLUTIONS FOR MAINTAINING CYANIDE EQUILIBRIUM WITH WATER AT 20° C. (APPROXIMATELY 10 PER CENT CALCIUM HYDROXIDE SUSPENSION INCLUDED IN EACH ONE)*

CONCENTRATION OF HCN IN WATER (M.)	CALCIUM CYANIDE CONCENTRATION FOR EQUILIBRIUM (M.)
0.010	1.41
0.0046	1.23
0.0022	0.87
0.0010	0.56
0.00046	0.31
0.00022	0.16
0.00010	0.089
0.000046	0.048
0.000022	0.026
0.000010	0.014
0.0000046	0.0074

*If carbon dioxide-free air is bubbled through the calcium cyanide solutions listed in the second column, it may then be passed through the hydrocyanic acid solutions listed in the first column without changing their concentrations.

which is produced. In Table I is listed a series of calcium cyanide-calcium hydroxide mixtures with their respective hydrogen cyanide equilibrium levels for either fresh or sea water at 20° centigrade.

In the cyanide experiments, a measurement of the oxygen consumption under control conditions is made first; then the proper quantity of a hydrocyanic acid solution one hundred times the desired final concentration is added to the water in the animal chamber through one of the top tubes. At the same time a clamp on a Y tube leading from the second chamber is closed to make the carbon dioxide-free air bubble through a tube containing 30 c.c. of calcium cyanide-calcium hydroxide mixture and then through a small tube of boric acid solution (which removes any ammonia that may be liberated from strong cyanide solutions). In this way the gas which returns to the animal chamber is always in equilibrium with the fluid in it, and even though the experiment is continued for hours, the concentration of the liquid will not change.

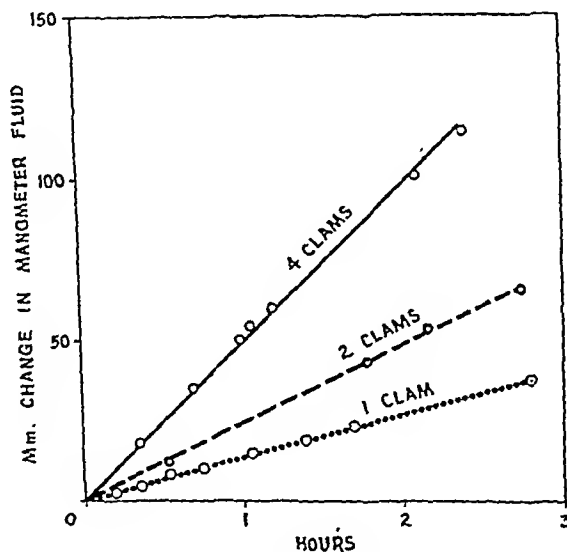


Fig. 2.—Respiration of the quahog, *Venus mercenaria*, as determined by the manometric respirometer. Ordinates indicate the change in the level of the manometer fluid at the times shown on the abscissas.

EXPERIMENTAL RESULTS

The apparatus has been tested in a variety of ways, and some of the experimental results are shown in Figs. 2 to 4. In Fig. 2 is illustrated the measurement of the oxygen consumption of the quahog or clam, *Venus mercenaria*. On the lowest curve is plotted the change in manometer reading with time when the chamber contained one animal in 1 liter of sea water. The middle curve shows the measurement when two animals were included, and the top curve that for four specimens.

In Fig. 3 are shown data from an experiment which demonstrates the effect of cyanide on the respiration of the quahog. Points along the upper curve were

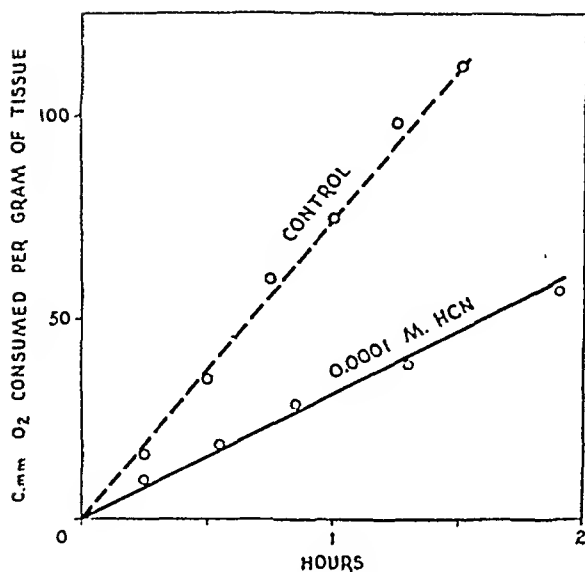


Fig. 3.—Oxygen consumption of an adult quahog during a control period and while it was immersed in 0.0001 M. HCN in sea water.

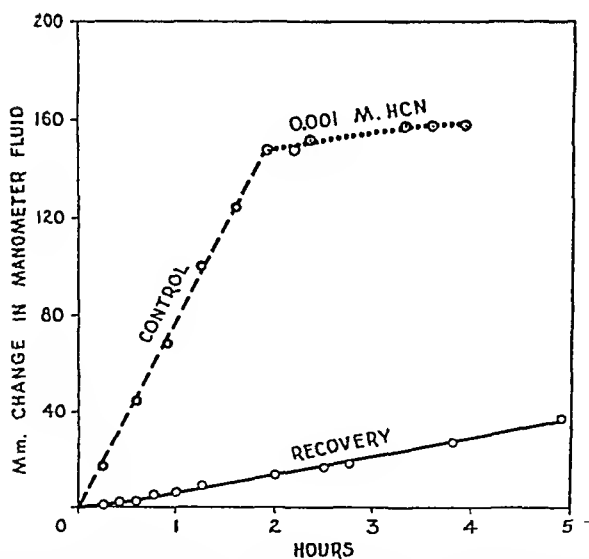


Fig. 4.—Manometric measurement of the oxygen consumption of a frog for control period during immersion in 0.001 M. HCN and for a recovery period after removal of the cyanide solution.

obtained during the control period. When enough 0.01 M. hydrocyanic acid was added to give a final concentration of 0.0001 M., the respiration fell off markedly as indicated by the lower curve.

In another experiment, a frog was allowed to sit on a platform half submerged in 1 liter of tap water. The curves in Fig. 4 show the course of the control respiration, the effect of adding cyanide (0.001 M. final concentration), and the course of the recovery after the water containing the cyanide was replaced with fresh water and the stream of hydrogen cyanide gas was shut off.

These experiments demonstrate the sensitivity of the method and illustrate the consistency of the results.

SUMMARY

An apparatus is described for making continuous measurements of the oxygen consumption of aquatic organisms. It is essentially a constant volume closed-circuit respirometer with a continuously flowing stream of air and manometric measurement of pressure changes. The sensitivity may be varied by adjustment of the volume of the system. It is useful in cyanide inhibition studies, for which purpose a table of calcium cyanide-calcium hydroxide mixtures that will maintain hydrogen cyanide equilibria is given. Illustrative experiments are reported.

The authors wish to thank Mr. Lee Allen for constructing the equipment.

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THE METHYLENE BLUE TEST FOR BILIRUBINURIA: CLINICAL AND SPECTROPHOTOMETRIC OBSERVATIONS

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FRANKE,¹ in 1931, described the methylene blue test for the detection of bilirubin in the urine. In 1940 Foord and Baisinger² made a comparative study of the Rosenbach, iodine, Müller, Huppert, Nakayama, Hunter, Naumann, Zins, Harrison, and methylene blue tests for bilirubinuria. They reported the methylene blue test to be as sensitive and as satisfactory as any of the nonconcentration methods.

The present study was prompted by the appearance in the literature in 1945 of two reports in which the methylene blue test was employed with usefulness. The first report is that of Myers,³ who used the test among employees working with tetrachlorethane and thereby subject to toxic hepatitis from such exposure. We were impressed with the finding that in some cases the result of the test became positive before the appearance of icterus and in some cases even prior to elevation of bilirubin in the serum. Also impressive was the report that the result of the test tended to become negative prior to return of the serum bilirubin to a normal level. The second report is that of Gellis and Stokes.⁴ The test was employed in seventy-seven cases of infectious hepatitis and in 1,000 cases in which normal persons served as control subjects. Of interest was the finding of a positive result in some of the cases of infectious hepatitis before the icterus index became elevated and the return to negativity as the icterus began to subside.

Figge⁵ has called attention to the fact that no chemical reaction takes place in the methylene blue test, since the test can be duplicated by holding a blue glass filter in front of a test tube containing either yellow urine or a solution of bilirubin. A tube containing a solution of bilirubin was immersed in a dilute solution of methylene blue. Figge noted the same green transmission band for the two superimposed but unmixed solutions as occurred when the solutions were mixed. He stressed the nonspecificity of the test by showing that penicillin, riboflavin, porphyrin, hemoglobin, or picric acid, when added to urine or water, will give false positive results for bilirubin.

Watson and associates⁶ likewise have indicated that the test is not specific for bilirubin, since yellow urines from normal persons and yellow substances such as potassium dichromate or ferric chloride will produce a green color when added to methylene blue. These authors also presented evidence to indicate that the test is due to a blend of color rather than to a chemical reaction.

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The following questions were suggested as we read the foregoing reports: Is the test specific for bilirubin, or will other substances produce a similar reaction? Is the green color of a positive result due simply to a blend of colors, or is it due to some type of chemical reaction? At what concentration of bilirubin in the urine and in the blood does the result of the test first become positive? Is there any correlation between the degree of hyperbilirubinemia and the positivity of the test? If the test is so delicate for bilirubin that it may show a positive reaction in urine before the serum bilirubin becomes elevated and may become negative while the patient is jaundiced and hyperbilirubinemia is still present, then might there be substances other than bilirubin which are responsible for the color reaction? Is the test of any value in estimating hepatic damage irrespective of the degree of hyperbilirubinemia?

MATERIAL AND PROCEDURES

It seemed of interest to observe the results of this test on urines from patients who had various types of disease affecting the liver and biliary tree sufficiently to produce jaundice or at least hyperbilirubinemia and particularly to study by means of the spectrophotometer the nature of the color reaction. For the test we have followed, in general, the procedure used by Gellis and Stokes.⁴ A 0.2 per cent aqueous solution of methylene blue chloride was freshly prepared. A pipette delivering approximately 20 drops per cubic centimeter was used. To 5 c.c. of urine (usually, but not always, collected before breakfast) were added 20 c.c. of tap water to make 25 c.c. of a 1:5 dilution. To this solution methylene blue was added drop by drop from the pipette until the initial green color of the mixture became blue. The result of the test was considered positive if 5 drops or more of methylene blue were required to change the color from green to blue. In most instances a check test was run in which methylene blue was added to 5 c.c. of undiluted urine until the green color changed to blue. The changes of color, particularly in darkly colored urines, often were less distinct by this method than by the dilution method. The latter method, therefore, was selected as the one of choice.

The clinical material on which this study was based consisted of twenty-eight patients who had conditions involving primarily the liver and the common bile duct. In all cases there was hyperbilirubinemia. In twenty-two cases jaundice was present.

RESULTS

In Table I is summarized the data for the twenty-eight cases. It should be noted that Cases 1 and 2 represent jaundice due to malarial treatment for syphilis; Cases 3 to 11 inclusive, jaundice due to hepatitis or cirrhosis; Cases 12 to 19 inclusive, jaundice due to benign stricture of the common bile duct or obstruction of the duct by calculi; Cases 20 to 23 inclusive, jaundice due to carcinomatous obstruction of the common bile duct; Cases 24 to 27 inclusive, jaundice of uncertain cause; and Case 28, jaundice due to familial hemolytic anemia.

TABLE I. SUMMARY OF DATA

CASE	DIAGNOSIS	BILIRUBIN (MG. PER 100 C.C. OF SERUM)		DROPS METHYLENE BLUE TO 25 C.C. URINE DILUTED 1:5	REMARKS
		DIRECT	INDIRECT		
1	Jaundice due to malarial treatment for syphilis	10.6	2.5	5	Ninth day of treatment
2	Jaundice due to malarial treatment for syphilis	2.2	1.2	2	Ninth day of treatment
3	Intrahepatic jaundice	10.6	1.0	6	Painless icterus, four months, recurrent
4	Residual hepatitis with jaundice	15.8	1.3	6	
5	Intrahepatic jaundice (chronic alcoholism)	38.6	7.8	16	Painless icterus, one week; dark urine, two months
6	Chronic hepatitis (residuum of infectious hepatitis)	25.1	1.8	14	
7	Chronic hepatitis (chronic alcoholism)	0.6	0.5	2	
8	Chronic alcoholism, cirrhosis of liver with ascites	1.6	1.3	4	
9	Chronic alcoholism, hepatomegaly	0	1.4	2	
10	Cirrhosis of liver with ascites	1.6	1.7	5	
11	Chronic hepatitis (chronic alcoholism)	19.6	2.9	20	
12	Cholecystitis and cholelithiasis, common duct stones, pancreatitis, hepatitis	6.2	0.8	5	
13	Subacute cholecystitis with stone, common duct stone, hepatitis	1.5	0.4	4	
14	Chronic cholecystitis with stones, common duct stone.	2.0		5	
15	Stricture of common duct, biliary cirrhosis	39.5	3.9	16	
16	Recurrent stricture of common duct	7.6	0.7	4	Tested three weeks postoperative
17	Recurrent stricture of common duct, biliary cirrhosis	4.0	0.7	4	
18	Stricture of common duct	15.3*	2.7	8*	
		3.2†		3†	
19	Stricture of common duct with external biliary fistula, chronic hepatitis	1.6	0.4	1	
20	Carcinoma of head of pancreas (three weeks after cholecysto-jejunostomy)	12.4	0.5	3	Bilirubin was 126 mg. per 100 c.c. of serum pre-operatively
21	Carcinoma of head of pancreas with hepatic metastasis	54.6	13.4	16	
22	Carcinoma of ampulla of Vater	29.9	1.4	9	
23	Carcinoma of pancreas (eleven days after cholecystogastrotomy)	17.6	1.1	4	Bilirubin was 54.6 mg. per 100 c.c. of serum pre-operatively
24	Hepatic metastasis, ascites, primary in pancreas (?)	47.6	3.6	25	
25	Painless jaundice 2.5 months, diffuse hepatitis (?)	54.6	1.8	15	
26	Postcholecystectomy syndrome (recurrent biliary colic with jaundice)	0	1.0	2	No cause found at operation
27	Intermittent painless jaundice one month (subsiding)	10.5	0.8	3	
28	Familial hemolytic jaundice	0	5.1	4	

*Preoperative.

†Postoperative.

In 1918 van den Bergh⁷ formulated the idea that the kidneys are impermeable at all times to indirect bilirubin, regardless of the concentration, but are permeable to direct-reacting bilirubin in concentrations of 2 mg. per 100 c.c. of serum or higher. In 1921 Lepehne⁸ and in 1925 McNee and Keefer⁹ confirmed this viewpoint. In 1933 Bensley,¹⁰ correlating the type and degree of hyperbilirubinemia with bilirubinuria, observed that with few exceptions bilirubinuria was present when the blood contained direct-reacting bilirubin in any concentration. With only a few exceptions bilirubinuria was not present when the blood contained only the indirect-reacting type of bilirubin regardless of the concentration. Lichtman¹¹ stated that the evidence from various sources indicates that the kidneys are impermeable at all times to indirect but are permeable to direct-reacting bilirubin in concentrations of 2 mg. per 100 c.c. of serum or more.

TABLE II. COMPARISON OF DEGREE OF BILIRUBINEMIA WITH POSITIVITY OF METHYLENE BLUE TEST

CASE	DIRECT-REACTING BILIRUBIN (MG. PER 100 C.C. OF SERUM)	NUMBER OF DROPS OF METHYLENE BLUE REQUIRED
<i>Group I. Negative Results (Less Than 5 Drops Methylene Blue)</i>		
9	0	2
26	0	2
28*	0	4
7	0.6	2
13	1.5	4
8	1.6	4
19	1.6	1
2	2.2	2
17	4.0	4
16	7.6	4
27	10.5	3
20	12.4	3
23	17.6	4
<i>Group II. Positive Results, Grade 1 (5 Drops Methylene Blue)</i>		
10	1.6	5
14	2.0	5
12	6.2	5
1	10.6	5
<i>Group III. Positive Results, Grade 2 (6 to 10 Drops Methylene Blue)</i>		
3	10.6	6
18	15.3	8
4	15.8	6
22	29.9	9
<i>Group IV. Positive Results, Grade 3 (11 to 15 Drops Methylene Blue)</i>		
6	25.1	14
25	54.6	15
<i>Group V. Positive Results, Grade 4 (16 or More Drops Methylene Blue)</i>		
11	19.6	20
5	38.6	16
15	39.5	16
24	47.6	25
21	54.6	16

*In Case 28, although the direct-reacting bilirubin was zero, the indirect was 5.1 mg. per 100 c.c. of serum.

In Table II the cases have been arranged into groups according to the degree of positivity of the result of the test and the concentration of bilirubin in the serum (direct-reacting type) for each in order to study the relationship of these two factors.

As may be noted in Table II, the correlation between the degree of hyperbilirubinemia and the degree of positivity of the methylene blue test is not perfect. The lack of perfect correlation is illustrated by a comparison of Case 22 and Case 11, in which the concentrations of bilirubin were 29.9 mg. and 19.6 mg. per 100 c.c. of serum, respectively. The urine in Case 22 required only 9 drops of methylene blue, while the urine in Case 11 required 20 drops to change the color from green to blue. Attention is called to Cases 16, 17, 20, 23, and 27 of Group I, in which the bilirubin ranged from 4 to 17.6 mg. per 100 c.c. of serum, and yet the methylene blue test gave a negative result (less than 5 drops of methylene blue). In Case 23 the methylene blue test was made postoperatively when the concentration of bilirubin was 17.6 as compared with a preoperative concentration of 54.6 mg. per 100 c.c. of serum. If bilirubin spills over into the urine at concentrations in the blood serum of 2 mg. per 100 c.c. or higher, and if the methylene blue test is as sensitive as reports²⁻⁴ imply, then it is hard to understand why the test gave negative results in the five cases mentioned. It might be that the renal threshold for bilirubin becomes altered in some manner as a result of severe and prolonged hyperbilirubinemia. Studies in this respect would be interesting.

TABLE III. COMPARISON OF METHYLENE BLUE AND NITRIC ACID TESTS

CONCENTRATION OF BILIRUBIN (MG. PER 100 C.C. OF URINE)	COLOR AFTER ADDING 4 DROPS METHYLENE BLUE TO 5 C.C. OF MIXTURE	GMELIN'S TEST
0	Blue	-
0.6	Blue	-
1.2	Blue	-
1.7	Blue	+
1.8	Blue	+
1.9	Blue	+
2.0	Blue green	+
2.1	Blue green	+
2.3	Green	+
2.5	Green	+
3.3	Green	+
5.0	Green	+

An attempt was made to determine the amount of bilirubin in the urine necessary to effect a positive result with methylene blue. Some of the pooled serum of jaundiced patients used in one of the experiments reported herein was added to normal urine to produce varying concentrations of bilirubin. Then these mixtures were treated both with methylene blue and with nitric acid on filter paper (Gmelin's test). The results are shown in Table III. The slightest trace of color in the Gmelin test became discernible at 1.7 mg. per 100 c.c. of urine. The result of the methylene blue test became positive when the concentration of bilirubin in the mixture was 2.0 mg. or more per 100 c.c.

of urine. It must be remembered that this is an artificial bilirubinuria produced by direct- and indirect-reacting bilirubin, and hence the results may not be applicable to the urine of a jaundiced patient.

SPECTROTRANSMITTANCE STUDIES

The spectrophotometric curves of urines giving positive results of the methylene blue test and other solutions used in the following experiments were determined by the use of a Coleman Junior Clinical Spectrophotometer, Model 6. With this instrument the percentage of light transmittance can be read at any wave length between 400 and 700 millimicrons. The data then are conveniently plotted on a graph with transmittance as the ordinate, expressed in percentage, and wave length as abscissa, expressed in millimicrons. Spectrophotometric curves of urines giving positive results of methylene blue tests were plotted. Each showed a curve characteristic of bilirubin with the maximal absorption of light between 425 and 450 millimicrons. The results of these spectrophotometric studies are shown in Figs. 1 to 5.

In Fig. 1 curve *a* represents the spectrophotometric curve of urine of a normal healthy man. Curve *b* shows the spectrophotometric curve of 5 c.c. of urine from the same person, to which has been added 2 drops of methylene blue to produce a blue color. The addition of the blue dye causes an absorption which is maximal at 660 millimicrons.

In Fig. 2 curve *a* represents the spectrophotometric curve of urine from a jaundiced patient, a man who had cirrhosis of the liver. The concentration of bilirubin on the day of this test was 25.1 mg. per 100 c.c. of serum direct, and 1.8 mg. per 100 c.c. indirect, by the van den Bergh reaction. The urine was dark brown and therefore had to be diluted before the methylene blue test could be read. Curve *b* is the spectrophotometric curve after 1 drop of the dye has been added to 5 c.c. of undiluted urine, yielding a clear green color. Curve *c* is the spectrophotometric curve of the solution obtained when 5 c.c. of the same urine were diluted with 20 c.c. of tap water and 14 drops of methylene blue were added to produce a blue color. Here is seen a wider band of absorption (from 600 to 675 millimicrons) than in curve *b* because of greater concentration of methylene blue.

In Fig. 3 curve *a* is the spectrophotometric curve of a solution of sodium bilirubinate with maximal absorption between 425 and 450 millimicrons. It is thus seen that a solution of sodium bilirubinate produces essentially the same transmittance curve as that of urine of a jaundiced patient (Fig. 2, curve *a*). Curve *b* represents the spectrophotometric curve of a solution of methylene blue in distilled water. There is a maximal absorption of 660 millimicrons. Curve *c* is the spectrophotometric curve of a green solution obtained by adding 4 drops of methylene blue to 5 c.c. of the sodium bilirubinate solution. There are two absorption bands, one from 425 to 450 millimicrons and the other at 660 millimicrons, showing that the green color is merely a blend of the two original colors. That there is no new substance formed is evidenced by the lack of any additional absorption band.

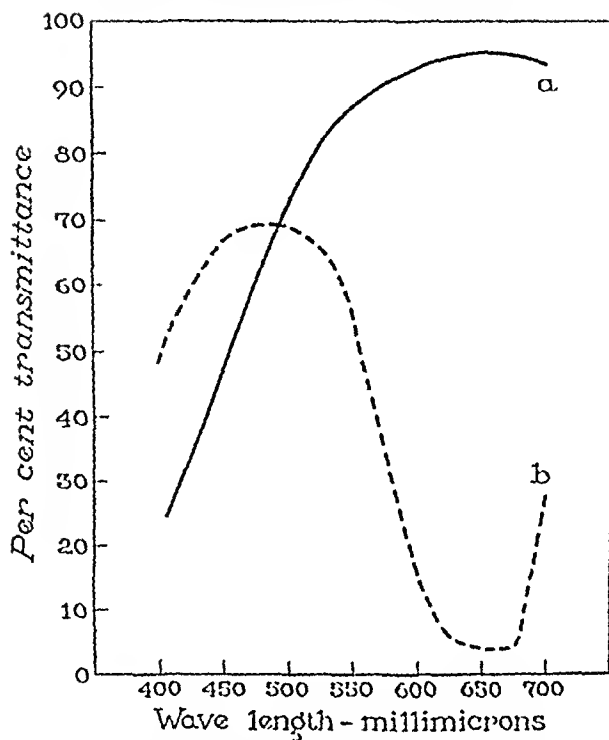


Fig. 1.—Spectrotransmittance (S-T) curves. Urine of a normal, healthy man: *a*, unmodified; *b*, with methylene blue added.

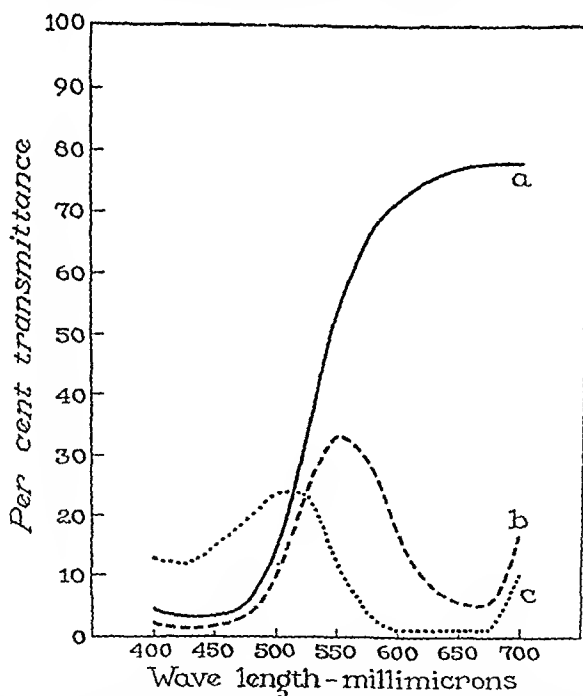


Fig. 2.—Spectrotransmittance (S-T) curves. Urine from a jaundiced patient: *a*, unmodified; *b*, with one drop of methylene blue added; *c*, with 14 drops of methylene blue added.

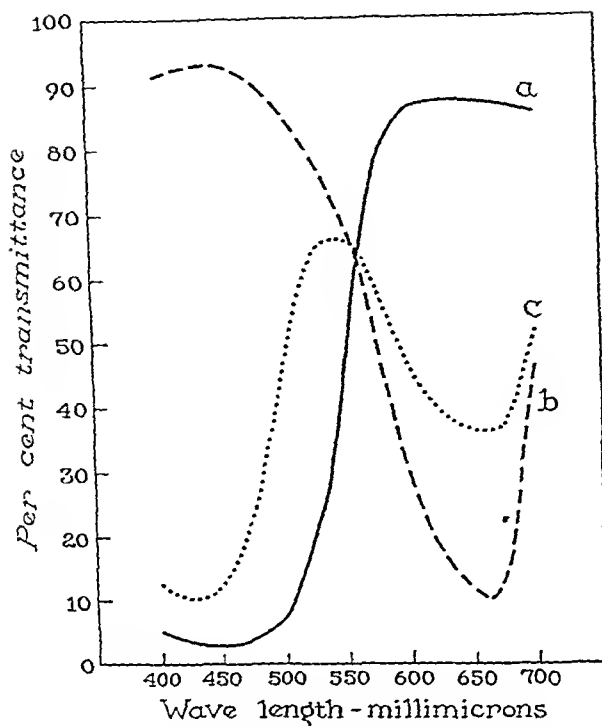


Fig. 3.—Spectrotransmittance (S-T) curves. *a*, A solution of sodium bilirubinate; *b*, a solution of methylene blue; *c*, a solution of sodium bilirubinate with methylene blue added.

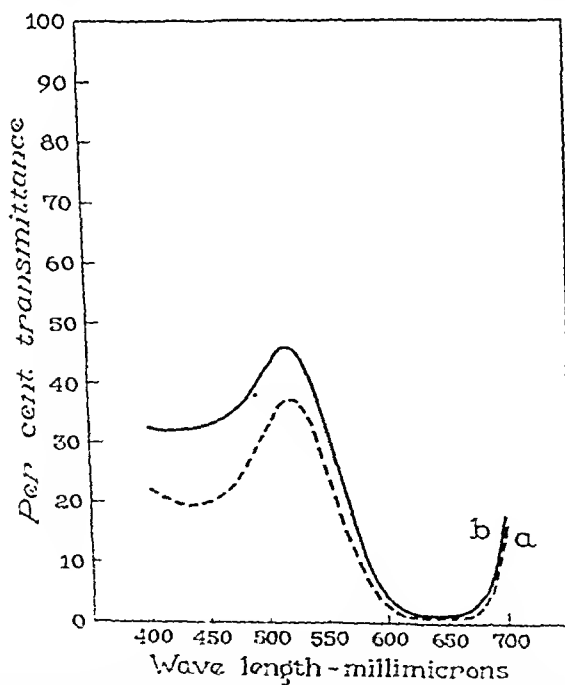


Fig. 4.—Spectrotransmittance (S-T) curves. *a*, Artificial bilirubinuria with methylene blue added; *b*, the same solution after further addition of methylene blue and further dilution with the same urine.

Fig. 4 represents the results of an experiment in which pooled serum containing 45.6 mg. per 100 c.c. direct- and 4.04 mg. per 100 c.c. indirect-reacting bilirubin was added to normal urine to give a concentration of 10 mg. of bilirubin per 100 c.c. of urine. Methylene blue was added drop by drop to produce a green—almost blue—color. The spectrophotometric curve of this solution is represented by curve *a*. Then more methylene blue was added to produce a definite blue color. This solution was then diluted with more of the normal urine, and the color returned to green. The spectrophotometric curve of the second green solution is represented by curve *b*. Because there has been no change in the curve, it may be reasoned that no new substance has been formed in the procedure.

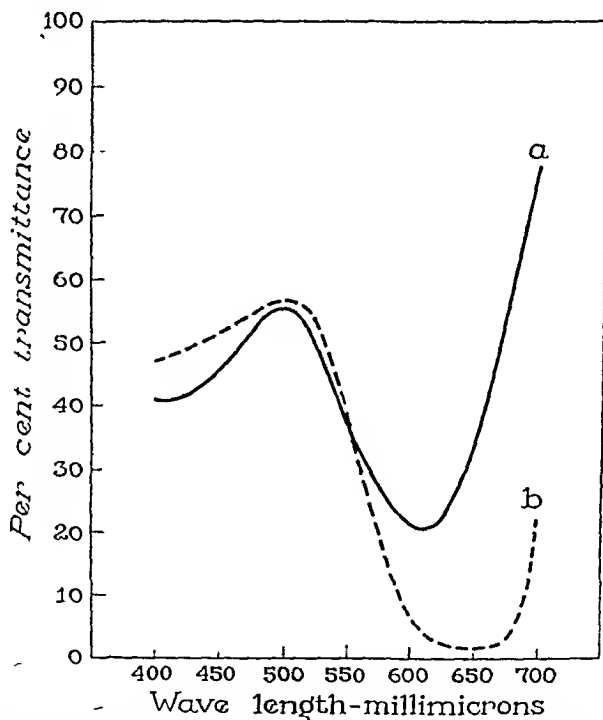


Fig. 5.—Spectrotransmittance (S-T) curves. *a*, Urine of jaundiced patients with Evans blue added; *b*, another specimen of the same urine with methylene blue added instead of Evans blue.

Urine for the determination, the results of which are shown in Fig. 5, was obtained from a patient who had jaundice due to stones in the common bile duct. A solution of Evans blue (T. 1824 dye) was made so that it had the same transmission of light as the methylene blue as measured by the spectrophotometer. Thus the solution of Evans blue and methylene blue were of the same intensity of color. Seven drops of Evans blue solution in 5 c.c. of urine were necessary to produce a blue color. The spectrotransmittance of the resulting blue solution is represented by curve *a*, with a maximal absorption at about 610 millimicrons. The urine was tested also with methylene blue, and 7 drops of the dye were required to produce a blue color in 5 c.c. of urine. The

spectrotransmittance curve of this solution is represented by curve *b*. This experiment tends to support the view that the color which results when methylene blue is added to urine which contains bilirubin is simply a blend of blue and yellow colors and can be duplicated by substituting a solution of Evans blue of the same intensity of color as the methylene blue.

SUMMARY

1. We did not observe perfect correlation between the concentration of bilirubin in the blood serum and the degree of positivity of the result of the methylene blue test.

2. The methylene blue test performed on urine with artificially produced bilirubinuria first became positive at a concentration of 2.0 mg. of bilirubin per 100 c.c. of urine.

3. Under the conditions of our experiments it would appear that the color which results when methylene blue is added to urine which contains bilirubin is due to a blend of blue and yellow colors rather than to some specific chemical reaction between methylene blue and bilirubin.

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A SIMPLE METHOD OF DETERMINING THE SPECIFIC GRAVITY OF SMALL SAMPLES OF URINE

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THE determination of the specific gravity of urine is a useful adjunct in judging renal function; however, it is often impossible to obtain a quantity sufficiently large for testing with an ordinary urinometer. Therefore, any method of determining specific gravity from a few drops of urine has possibilities of wide application, especially when it does so without requiring the use of a timing device or chart for interpretation.

The method described in this paper is a combination of two well-known techniques, that of Barbour and Hamilton¹ and that of Phillips and associates.² As in testing by the copper sulfate technique of Phillips, drops of urine are observed in insoluble media of known specific gravities, and, as in that method, there is no need to use a watch. Furthermore, the method herein presented can be used to measure the specific gravity of all watery solutions, as can that of Barbour and Hamilton. It does not require adjustment of mixtures while the test is in progress, as does the procedure of Kirkpatrick and Kling.³

For this determination a series of specific gravities covering the desired range may be prepared in 100 c.c. lots by mixing xylene and bromobenzene (XBB)* in the proper proportions. Solutions may be made either volumetrically according to the directions given in Table I or roughly by trial with a urinometer as a guide. The urine is introduced most easily under the surface of successive solutions in the graduated series by use of an ordinary dropper, the drops being displaced from the tip by an upward motion. The specific gravity of the urine lies between that of the mixture in which the drop rises and that in which it falls; or, if the drop neither rises nor falls in the bottle, the specific gravities of the urine and mixture are identical.

With ideal temperature control of numerous accurately prepared XBB mixtures, this method can be made equal in accuracy to the falling drop determination of Barbour and Hamilton.¹ Even using a roughly graded series and with ordinary fluctuations of temperature, this method is as accurate as regular determinations with a hydrometer. A series of mixtures covering the range from 1.005 to 1.029, inclusive, in rough fashion (that is, 1.005, 1.010, 1.015, 1.020, 1.025, 1.029) will give sufficient information to be of clinical value. For improved accuracy, slight changes in specific gravity due to evaporation of the mixtures can be minimized by corking the bottles tightly and can be corrected by the appropriate addition of xylene or bromobenzene. When XBB mixtures are used at temperatures widely variant from that at which they were prepared, correction should be made. Thus, the specific gravity should be changed

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*Eastman Kodak, Xylene T275, Bromobenzene No. 13.

TABLE I

SPECIFIC GRAVITY	XYLENE (C.C.)	BROMOBENZENE (C.C.)
1.005	78.18	21.82
1.010	77.38	22.62
1.015	76.58	23.42
1.020	75.78	24.22
1.025	74.98	25.02
1.029	74.34	25.66

Thus, a 0.16 c.c. increase in the quantity of bromobenzene and an equal decrease in the amount of xylene will increase the specific gravity .001.

.0008' for each degree centigrade deviation in observed temperature from the calibration temperature (that is, if the XBB mixtures were originally prepared at 22° C. and the temperature at the time the test is made is 17° C., the specific gravity in each of the bottles should be corrected by adding .004).

After repeated use the mixtures may become cloudy so that observation of the progress of the drops is difficult. This is due to the accumulation of urinary sediment plus the excess of urine in the bottles. The mixtures may be easily cleaned by passing each through an average-grade filter paper, and the urine may be largely removed by decanting or by using a small separatory funnel.

This method, while useful in lieu of a urinometer in all determinations of the specific gravity of urine, has several specialized applications. The urologist, when called upon to test separately and comparatively a patient's kidneys, will find this a most sensitive technique to use in addition to the relatively crude phenolsulfonphthalein test; for with but a few drops of urine obtained from each ureteral catheter, he can measure accurately the tubular concentrating power of an individual kidney. Thus, a differential dehydration test for comparison of renal function is made possible. The pediatrician will find the method of value for determining the specific gravity of urine of babies and children whose specimens are scanty. Studies of renal function in small animals also will be facilitated, as the specific gravity of urine produced by a single animal may be measured and reliance on pooled specimens eliminated.

SUMMARY

A simple method for determining the specific gravity of small samples of any aqueous solution is presented for use in clinical and laboratory work.

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which are carried by the official Journal are not only abstracted but so restated without any factual change that the lay public can read and digest this basic scientific, economic, and sociological material. It is the hope of the Gerontological Society, as well as the editor of this Journal, that they may reach an ever expanding and varied order of reader interested in the general theme of ageing.

Volume 1, Number 1, January, 1946, of *The Journal of Gerontology* carries such varied contributions as the "Increase in Mortality as a Manifestation of Ageing" by Henry A. Simms, of the Department of Pathology of Columbia University Medical School; "Ageing in Nutritionally Deficient Persons" by Tom D. Spies and Harvey S. Collins, of the Nutrition Clinic, Hillman Hospital, Birmingham, Alabama; "Budgeting for Social Security" by W. R. Williams, Actuarial Consultant, Federal Security Agency; and "Shakespeare's Attitude Towards Old Age" by John W. Draper, Department of English, University of West Virginia.

The primary object of *The Journal of Gerontology* is to publish scientific papers, the products of research, which have as their objective an understanding of those changes of a chemical and physical order acquired by tissues as they advance from the commencement of the life of the organism through successive age segments towards senility and senile death. With such information this Journal would also have the high hope "to add life to years, not just years to life."

WM. DEB. MACNIFER.

THE EXPERIMENTAL PRODUCTION OF GENERALIZED ARTERITIS AND PERIARTERITIS (PERIARTERITIS NODOSA)

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AMONG the various etiologies suggested for periarteritis nodosa, allergy has had a prominent place since Gruber¹ first suggested its possible causative role in certain cases which he observed. Recent clinical-pathologic studies by Rich^{2, 3} have strengthened this view. Furthermore, Rich and Gregory⁴ have shown that lesions characteristic of periarteritis nodosa can be produced in rabbits by the intravenous administration of large doses of sterile, normal, horse serum. Such lesions followed a single dose but were seen more regularly after multiple injections of the foreign serum. The significance of these observations is increased by the fact that lesions similar in many respects to those of rheumatic pancarditis were produced in the course of their experiments.^{5, 6}

The clinical correlation of periarteritis nodosa with allergic phenomena of a different type has been made recently by Wilson and Alexander,⁷ who found that bronchial asthma was present in fifty-four of 300 consecutive cases of this disease. Such a relationship is not apparent in all reports dealing with the same problem, however, and it has been stated that convincing evidence which points to any one responsible etiologic agent is lacking. Harris, Lynch, and O'Hare,⁸ in a review of the recorded clinical cases of periarteritis nodosa, discussed those conditions which at one time or another have been considered as causative. In addition to an allergic mechanism these included such infections as may be produced by *Treponema pallidum*, streptococci, viruses, and protozoa. They stressed the point that the clinical course of periarteritis nodosa resembles that of a generalized infection with toxemia. The fact that a history of allergic disease was obtained in 15 per cent of their collected cases failed to impress them. More recently, Jones⁹ has reviewed this subject and has considered either allergy, rheumatic fever, or infection of many types as a significant predisposing factor. In his opinion, however, none of these emerges as the dominant factor.

Thus, although periarteritis nodosa is a distinct entity on the basis of its characteristic gross and histologic features, it seems impossible, clinically, to attribute to it a single etiology. It becomes of primary importance then to seek the mechanism of development of this peculiar arteritis. Although this pathologic process may be affected by a variety of apparently unrelated cir-

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cumstances, there may yet be a single *pathogenesis*. Recent experimental data give support to this view. The studies of Rich and Gregory, as well as the ones which we shall describe, leave little doubt that the intravenous administration of large amounts of horse serum to rabbits can lead to a necrotizing and proliferative arteritis which, in both its acute and chronic stages, meets the essential microscopic criteria of periarteritis nodosa.

Using an entirely different approach, other investigators¹⁰⁻¹² have indicated recently that hypertension, experimentally induced, also may lead to a focal necrotizing arteritis and periarteritis. Smith, Zeek, and McGuire¹³ have reviewed these and other similar reports. They have drawn attention to the fact that in all of these experiments, which include several species of animals (dogs, rats, and rabbits), either hypertension or renal disease were present. They record the occurrence of periarteritis nodosa in twenty-six of sixty-two rats and in four of eight dogs which they themselves made hypertensive. In their experiments hypertension was the result of a perinephritis induced by wrapping one kidney with silk. Seyle and Pentz¹¹ also produced lesions similar to those of periarteritis nodosa in rats made hypertensive by a somewhat different procedure. Following unilateral nephrectomy the animals were given large injections of desoxycorticosterone acetate; this was accompanied by an increased oral intake of sodium chloride. On the basis of experimental as well as clinical data, it would appear then that there is no single *etiology* for these focal arterial lesions.

The following experiments represent, first, a repetition of those of Rich and Gregory which, to the present time, apparently have not been repeated by other investigators. Second, considerable new data are presented which include serologic studies during the development of the various allergic lesions in the experimental animals. Third, certain aspects of our experiments have been designed to determine the underlying mechanisms responsible for the production of periarteritis nodosa.

MATERIAL AND METHODS

Sixteen white male albino rabbits, weighing from 2.7 to 3.9 kilograms and housed in individual wire-bottomed cages, were used. They were maintained on a commercial rabbit food* and water ad libitum, with the addition of approximately 150 Gm. of carrots two times per week. The horse serum used was sterile normal serum, without preservative, and was pooled from two sources.† A 10 per cent suspension of sterile powdered sulfadiazine was prepared in a 10 per cent solution of gum acacia in order to facilitate precise oral administration. This relatively stable suspension was administered by means of a stomach tube.

Rectal temperatures were determined at approximately 9 A.M. daily from the day before the first injection of horse serum through the twenty-sixth day of the experiment. The ears of the animals were carefully inspected daily from the fifth to the tenth day of the experiment and from the eighteenth to the twenty-fifth day for evidence of erythema and edema of the type originally described by Fleisher and Jones¹⁵ as characterizing serum sickness in rabbits. Cutaneous reactions to 0.1 c.c. of horse serum or 0.1 c.c. of a suspension of sulfadiazine, injected intradermally, were observed on the eleventh and twenty-sixth days of the experiment. The sites of inoculation were inspected at one hour and at twenty-four hours.

*Alpha Flakes.

†Kindly donated by the Research Laboratories of Eli Lilly and Co., Indianapolis, Ind., and Lederle Laboratories, Inc., Pearl River, N. Y.

Antibody titers to horse serum were determined using the collodion particle agglutination technique of Cannon and Marshall.¹⁶ Simultaneously, and using the same samples of serum, we attempted to demonstrate humoral antibodies to sulfadiazine by means of the serologic "inhibition" reaction. This was accomplished by making up a second series of dilutions of the antisera, using as the diluent a saturated solution of sulfadiazine in 0.86 per cent saline. This procedure allowed opportunity for the antiserum to combine with the sulfadiazine before the horse serum-coated particles were added. Tissues for histopathologic study were fixed in Zenker's solution, imbedded in celloidin, and stained with hematoxylin and eosin, Mallory's connective tissue stain, azan carmine, and Masson's trichrome stain (Goldner's modification).

EXPERIMENTAL PROCEDURES AND RESULTS

The animals were divided into two groups. Rabbits 1 to 10 received horse serum alone, while Rabbits 12 to 17 received sulfadiazine in addition to the horse serum (see Table I). It should be stressed that the type of experimental animal, various materials, dosages, and time intervals, as related to the injections of horse serum and sulfadiazine, followed, in so far as possible, those of Rich and Gregory.⁴ For reasons which will be discussed later, the rate of injection of the initial dose of horse serum was varied as indicated in Table I.

TABLE I. TIME, AMOUNT, AND MODE OF ADMINISTRATION OF NORMAL HORSE SERUM AND SULFADIAZINE AND TIME OF DEATH

RABBIT No.	Kg.	DAY OF EXPERIMENT																																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	
1	3.9	A															1	X																		
2	3.6	A															1		A									X								
3	3.8	A															1		A									X								
4	3.4	A															1		A																2	X
5	2.7	A															1		X																	
6	3.7	B															1	X																		
7	3.7	B															1		A																2	X
8	3.7	B															1		A									X								
9	3.5	C															1		A									X								
10	3.7	D															1		A									X								
12	3.4	A															X																			
13	3.1	A															1																		X	
14	3.0	A															1		X																	
15	3.2	A															1		A																X	
16	3.3	A															1																			
17	3.1	A															1		A																X	

LEGEND: A=10 ml. H.S./kg. (i.v.)-10 min. X=Animal killed. ⊗=Animal Died Spontaneously.
 B=same dose-3 equally divided portions inj. over 8 hour period.
 C=same dose inj. in 5 min. —=Sulfadiazine, 0.5 gm/kg. (by mouth) per day.
 D=same dose inj. in 2½ min. ⊙=Sodium Sulfadiazine 1.0 gm. (i.v.)
 ①=1 ml. H.S. (i.v.) ②=2 ml. H.S. (i.v.)

The rise in temperature¹⁷ and the erythema and edema of the ears¹⁵ characteristically exhibited by rabbits following injection of large doses of horse

TABLE II. DEGREE OF VARIOUS REACTIONS OBSERVED FOLLOWING ADMINISTRATION OF NORMAL HORSE SERUM (AND SULFADIAZINE) TO ALBINO RABBITS

RABBIT	TEMPERATURE RISE* (FOURTH TO NINTH DAY)	EAR REACTION† (FIFTH TO TENTH DAY)	ARTHUS REACTION‡ (ELEV-ENTH AND TWELFTH DAYS)	PRECIPITIN TITER§ (FIFTEENTH DAY)	TEMPERATURE RISE* (TWENTIETH TO TWENTY-FIFTH DAY)	EAR REACTION‡ (TWENTIETH TO TWENTY-FIFTH DAY)	ARTHUS REACTION‡ (SIXTH AND TWENTY-SEVENTH DAYS)	PRECIPITIN TITER§ (TWENTY-SIXTH DAY)	VASCULAR LESIONS AT DEATH¶
1	1+	±	1+	480	—	—	—	—	±
2	3+	1+	3+	480	3+	0	—	960	3+
3	1+	3+	3+	480	4+	0	—	1,920	2+
4	2+	1+	2+	960	3+	±	3+	960	2+
5	2+	0	1+	960	—	—	—	—	3+
6	0	3+	3+	480	—	—	—	—	2+
7	1+	±	2+	960	3+	±	2+	1,920	1+
8	2+	0	2+	960	1+	0	—	960	3+
9	2+	1+	3+	480	3+	0	—	960	4+
10	1+	4+	2+	480	3+	±	—	480	1+
12	0	1+	1+	480	—	—	—	—	±
13	1+	0	2+	240	1+	0	1+	480	±
14	0	0	2+	480	—	—	—	—	±
15	2+	±	3+	960	—	—	—	—	1+
16	1+	0	2+	480	4+	0	—	—	±
17	0	1+	1+	240	0	0	1+	240	±

0, Negative; —, not determined; ±, doubtful positive reaction.

*The average temperature during this period was compared with the average of a preceding five-day control period. An increase of less than 0.1° F., 0; 0.1 to 0.5° F., 1+; 0.5 to 1.0° F., 2+; 1.0 to 1.5° F., 3+; over 1.5° F., 4+, 5+.

†This evaluation was entirely subjective and was based on the intensity of the erythema and edema and its duration.

‡Hyperemia with little or no edema, 1+; hyperemia with moderate to marked edema, 2+; marked hyperemia and edema with hemorrhage and central necrosis, 3+.

§Using the colloidal particle agglutination technique (serial dilution of antibody).

¶After the tissues had been thoroughly studied microscopically, grading of the vascular lesions in each animal was accomplished by means of subjective analysis based on the intensity and frequency of these lesions.

§The cause of this temperature rise is questionable. During this period the animal appeared sick; at death there was marked obstructive nephropathy (sulfadiazine) and marked pulmonary edema.

**These animals died spontaneously.

serum, as well as the Arthus reaction and precipitin titers to horse serum, were carefully studied in an attempt to determine the extent of hypersensitivity exhibited by the animals at various intervals following the injections of horse serum. At the conclusion of the study the quantitative character of these ante-mortem reactions was compared with the intensity and frequency of the vascular lesions observed. In order to facilitate this comparison, the results have been condensed and tabulated in Table II. Of these data several points are particularly noteworthy. It is quite apparent that the animals receiving both sulfadiazine and horse serum, as compared with those which received the same doses of horse serum alone, exhibited a markedly lowered precipitin titer, temperature response, etc. Not only were these allergic and serologic reactions to horse serum consistently less, as judged by the observations made before death, but the frequency and intensity of the vascular lesions were also much less in the group that received horse serum and sulfadiazine. It appears thus that there is a gross correlation between antibody response and the extent and degree of arteritis following the injection of large

amounts of horse serum. When either of the groups is studied alone, however, it becomes apparent that there is no consistent correlation between ear reactions, Arthus reactions, or precipitin titers and the type or extent of vascular response apparent at autopsy. The temperature rise which occurred following the first injection of horse serum, in the animals receiving horse serum alone, did tend to parallel the degree and extent of arteritis observed at death. We cannot explain the marked diminution in ear reactions following the second large dose of horse serum, unless the reactions were all of the "immediate" type¹⁶ and hence so transient as to escape our daily observations.

In addition to the data recorded in Table II, skin tests performed with a 10 per cent suspension of sulfadiazine in saline on the eleventh day of the experiment were negative in Rabbits 12, 15, and 17 but were doubtfully positive in Rabbits 13, 14, and 16. In these latter animals a 5 by 5 mm. area of edema and slight erythema was observed at twenty-four hours, whereas similar intracutaneous injections in four animals which had received no sulfadiazine previously produced no reaction. Attempts to demonstrate circulating antibodies to sulfadiazine by means of the serologic "inhibition" reaction, utilizing serum obtained on the fifteenth day of the experiment, were completely unsuccessful. Under these conditions, inhibition of the precipitin reaction might have been expected, if antibodies had been formed to a sulfadiazine-horse serum complex.

Yet another ante-mortem observation of considerable interest was the appearance of a maculopapular erythematous eruption over the abdomens of four rabbits following the second large injection of horse serum (nineteenth day). The hair had been clipped nine days earlier in preparation for skin testing; at the time of the erythematous response the Arthus reaction had subsided completely. This eruption appeared in Rabbits 2 to 4 from the twenty-first to the twenty-third day and was seen on the twenty-second and twenty-third days in Rabbit 9, following which it faded rapidly. The erythema was marked, and there was no indication of trauma nor other extraneous stimulus as a cause. Why the ears of the rabbits did not react at the same time is difficult to explain, but the time of appearance and disappearance of this rash corresponds so well with that of "accelerated serum sickness" in rabbits,¹⁸ which might be expected following this second injection of horse serum, that it is difficult for us to dismiss the lesions as coincidental. If the aurai erythema and edema described by Fleisher and Jones¹⁷ is analogous to human serum sickness, one would expect that it would not necessarily be localized to the ear of the rabbit. A biopsy of this dermal lesion from Rabbit 2 was obtained on the twenty-third day of the experiment. Microscopic examination revealed a superficial dermatitis characterized by moderate diffuse infiltration of leucocytes, edema, and a pronounced hyperemia of the small veins. Most of the infiltrating cells were mononuclear, although polymorphonuclear leucocytes were present. In the subdermis the collagenic fibers were swollen and vacuolated and occasional areas exhibited fibrinoid necrosis. The inflammatory process diminished in intensity in the subcutaneous region where it was localized about small blood vessels, chiefly veins (Fig. 1).

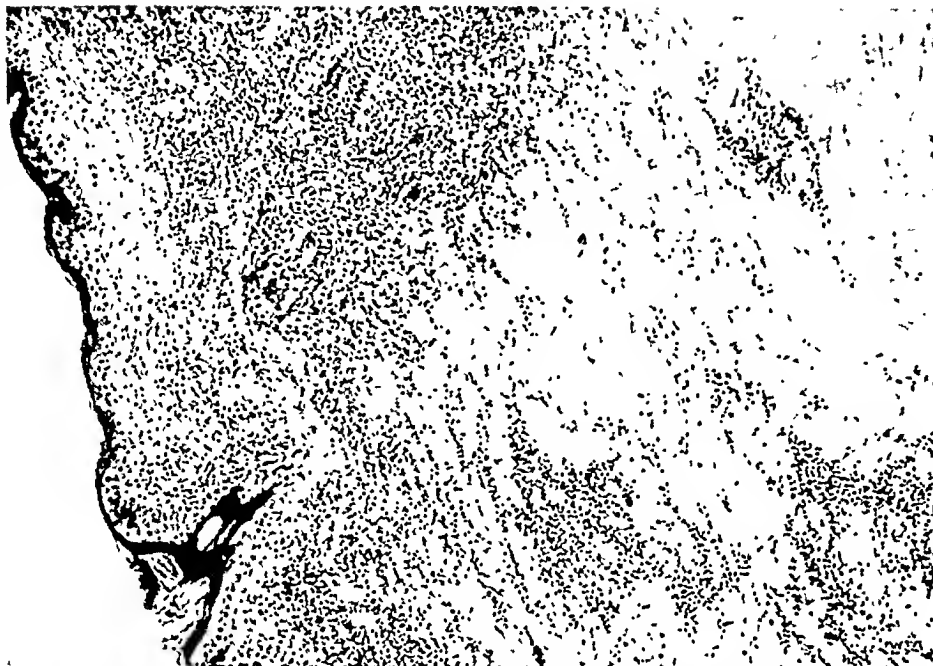


Fig. 1.—Rabbit 2, twenty-third day. Biopsy of erythematous eruption of skin showing infiltration by lymphocytes, focal areas of necrosis, edema of the superficial dermis, and hyperemia ($\times 125$).



Fig. 2.—Rabbit 9, twenty-sixth day. Section of a coronary artery showing marked periarteritis and necrotizing arteritis with almost complete occlusion of the lumen by infiltrating cells, most of which are lymphocytes and macrophages ($\times 155$).

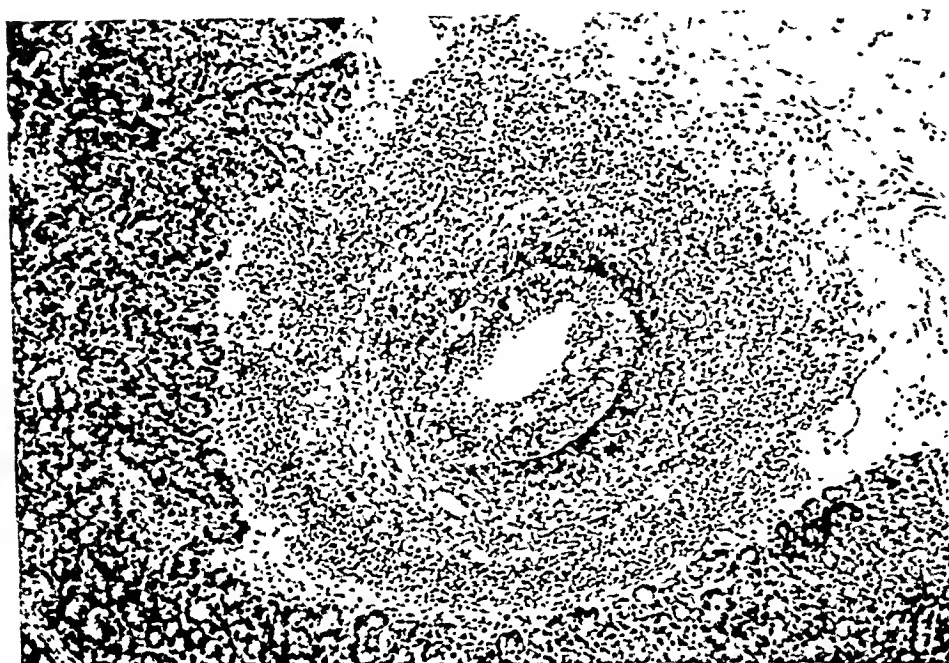


Fig. 3.—Rabbit 9, twenty-sixth day. Section of the pancreatic artery showing marked fibrinoid necrosis of the media accompanied by a marked arteritis and periarteritis ($\times 130$).

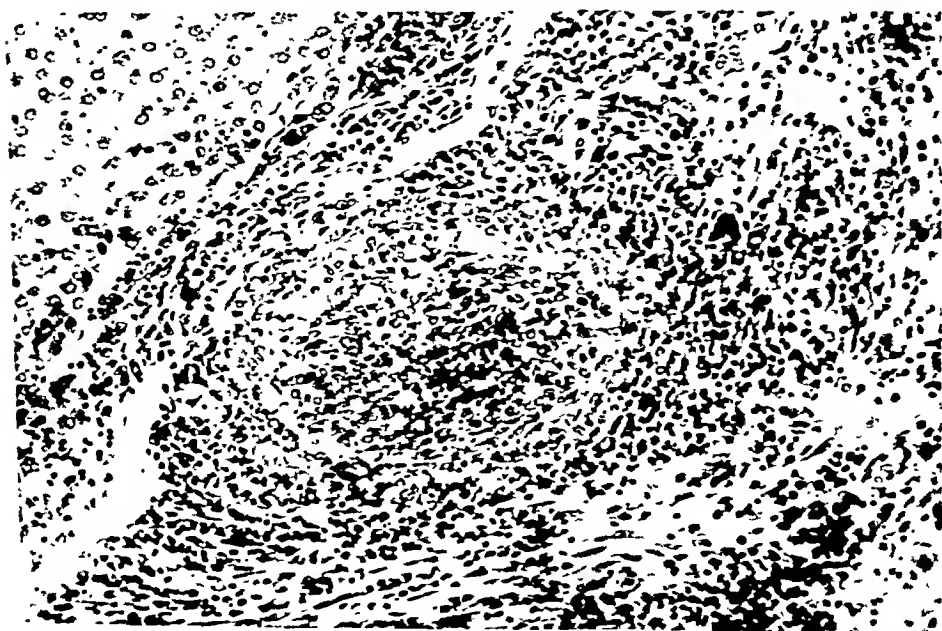


Fig. 4.—Rabbit 9, twenty-sixth day. Section of an hepatic artery with a similar arteritis and periarteritis. The lumen has been reduced to a small slit ($\times 285$).

POST-MORTEM FINDINGS

Gross Findings.—Moderate to marked enlargement of the spleen was the most constant gross finding. In addition, four of the six animals (Rabbits 12 to 15) receiving sulfadiazine showed moderate to marked enlargement of the kidneys. Cut surfaces exhibited a yellow-white granular precipitate arranged in strands paralleling the long axis of the pyramids. In four animals (Rabbits 3, 12, 14, and 16) the thymus was markedly atrophic. Two animals of the group (Rabbits 13 and 16) receiving sulfadiazine presented marked pulmonary edema and hyperemia. These animals were the two which had received sodium sulfadiazine intravenously on the nineteenth day; they died unexpectedly on the twenty-eighth and twenty-fourth days, respectively. Slight parasitic infection of the liver or mesentery was observed in four animals (Rabbits 1, 8, 9 and 14). In one case the organisms were identified as encysted tapeworms and in another as coccidia. None of the arterial lesions described below was grossly apparent, and there were no obvious manifestations such as thrombosis, infarction, aneurysm formation, or hemorrhage which might have suggested their presence.

Microscopic Findings.—

Heart and Blood Vessels: Vascular inflammatory changes similar to those described by Rich and Gregory were the most frequent lesions encountered. Only one animal (Rabbit 9), however, showed a marked *generalized* necrotizing arteritis (Figs. 2 to 4). These lesions were seen in the small and medium sized arteries of the heart, liver, pancreas, spleen, thymus, and lymph nodes. Some of the larger arteries showed focal necrosis and acute inflammation of their walls (Fig. 5). Acute and subacute periarteritis, characterized by polymorphonuclear and/or mononuclear leucocytic infiltration of the adventitia, was seen in eleven of the sixteen animals (Figs. 6 and 7). Proliferative lesions of the endothelium and chronic inflammatory changes of the media and adventitia were commonly observed (Rabbits 2, 7, 9, 10, and 14). In these, the cellular infiltrate was composed of lymphocytes and macrophages predominantly, and there was usually an associated medial and periarterial fibrosis of varying degree. The earliest changes appeared to be a "loosening" of the adventitial connective tissue with edema and a delicate infiltration of monocyte cells. Accompanying this, a similar change in the medial layer was often observed. This apparently preceded the fibrinoid necrosis and actual disruption of the media. Accompanying the necrosis there was proliferation and intercellular edema of the endothelium. At this stage cellular infiltration was often very marked, and there was an almost complete obliteration of the vascular lumen (Figs. 2 and 4). Fibrosis of the media and adventitia appeared to be a sequel to this. These various stages of arteritis were most frequently seen in the heart, although they were observed in other organs. This distribution is similar to that reported by Vaubel¹⁹ and by Miura²⁰ who produced arterial lesions in rabbits by repeated injections of foreign serum. Focal periphlebitis of a slight degree was observed in several instances.

Lesions with some of the characteristics of Aschoff bodies were encountered occasionally (Fig. 8). Unfortunately, our observations did not include

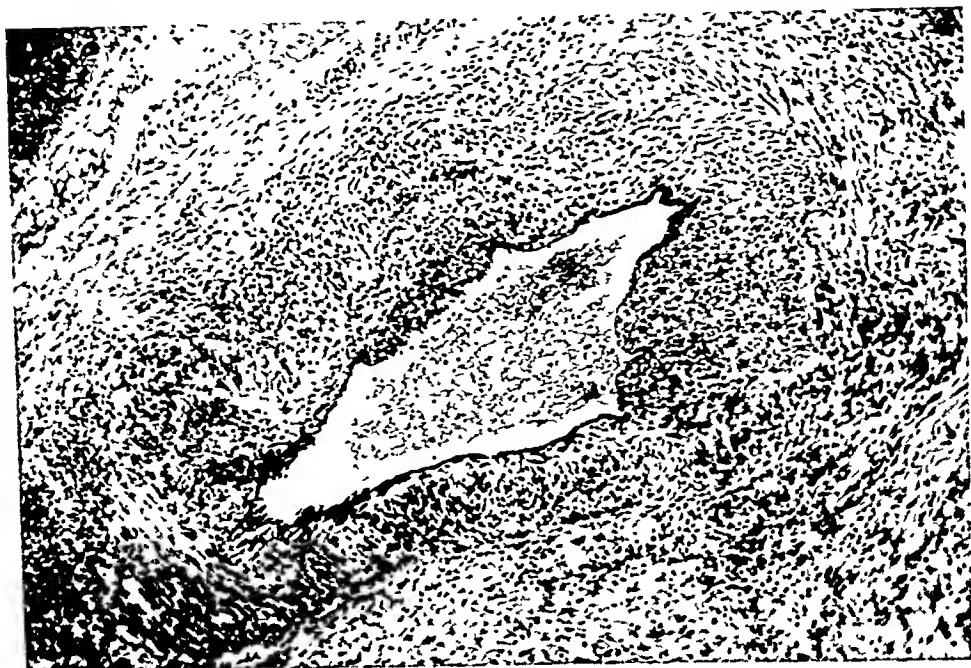


Fig 5.—Rabbit 9, twenty-sixth day. Focal necrosis, arteritis, and periarteritis of a large artery near the thymus ($\times 120$).

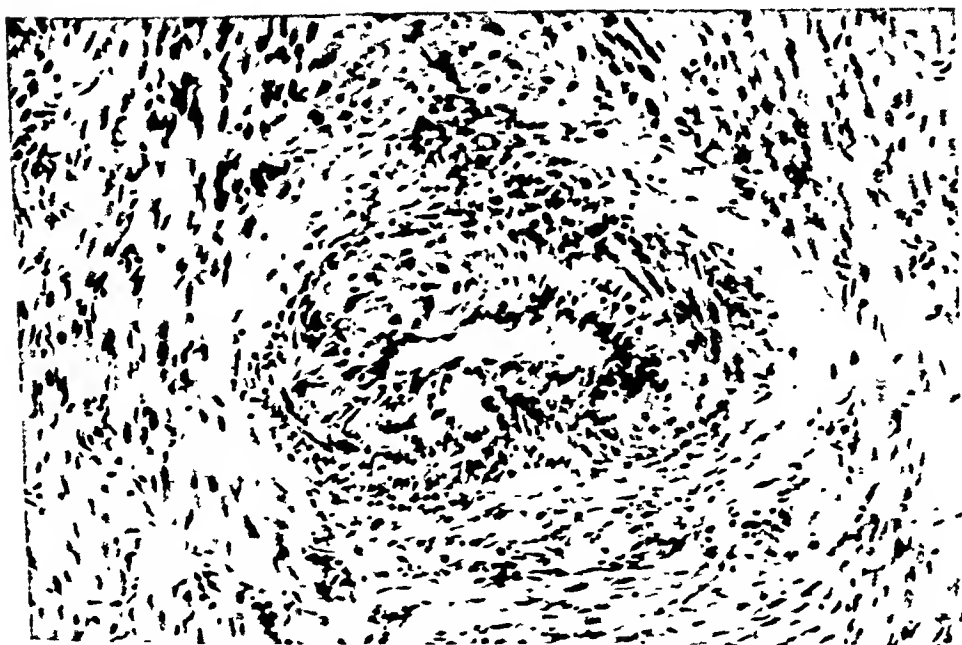


Fig 6.—Rabbit 4, thirty-fourth day. A small branch of a coronary artery showing periarteritis. Although most of the inflammatory cells are lymphocytes, polymorphonuclear cells are present ($\times 235$).

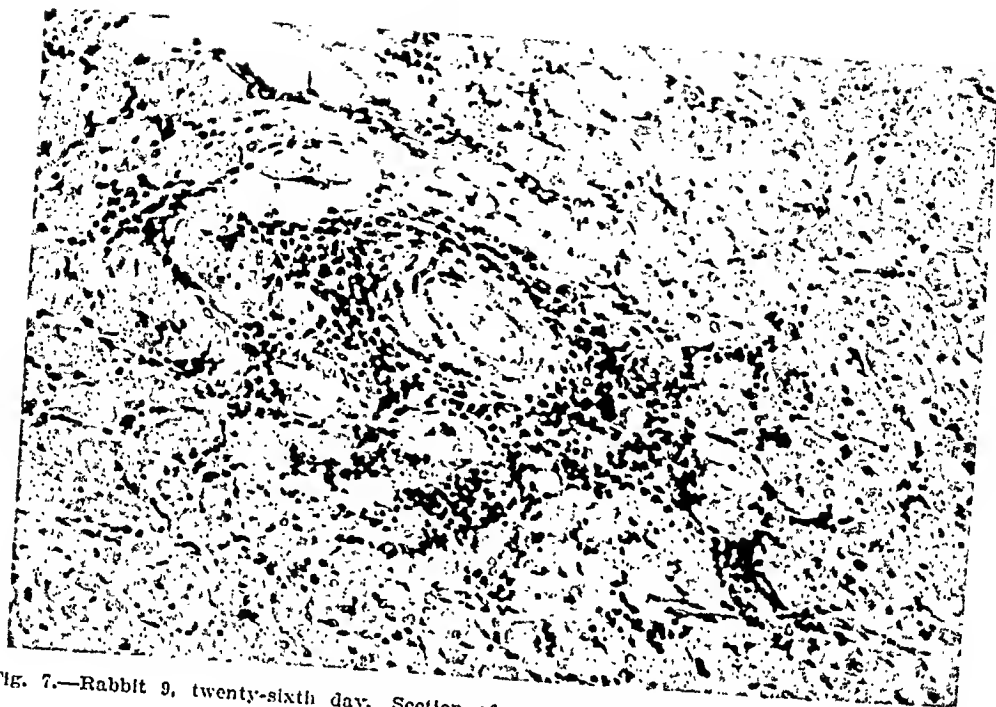


Fig. 7.—Rabbit 9, twenty-sixth day. Section of myocardium showing round cell infiltration around a small artery (X265).



Fig. 8.—Rabbit 8, twenty-sixth day. This perivascular lesion in the myocardium resembles an Aschoff body (X450).



Fig. 9—Rabbit 14, nineteenth day. Section of the thoracic aorta showing cystic necrosis of the media. This lesion was present throughout the entire circumference at this level ($\times 275$).

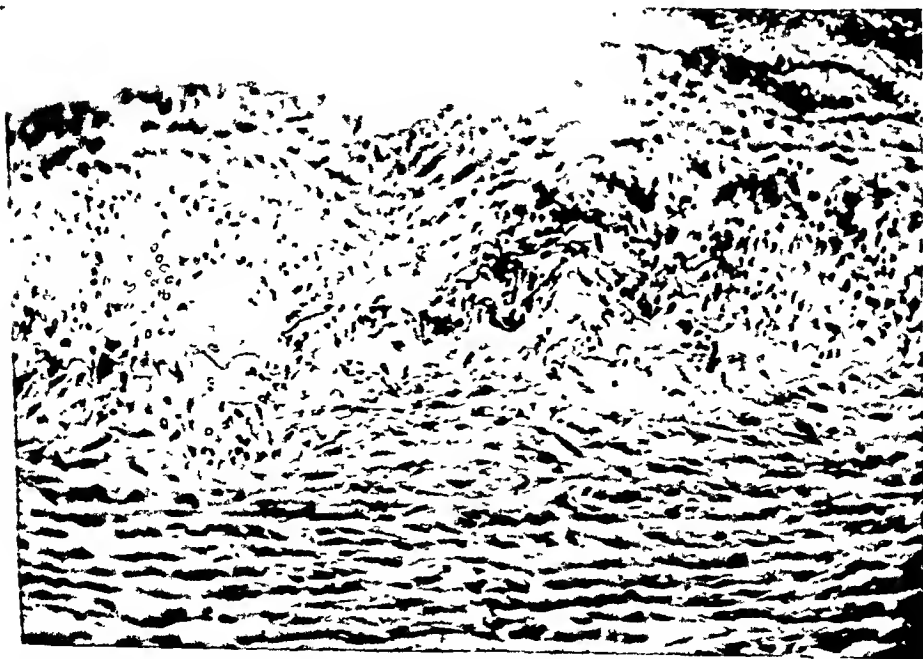


Fig. 10—Rabbit 15, twenty-first day. This focal area of cystic medial necrosis lies in the thoracic aorta. Note the fibrinoid necrosis and infiltration of large mononuclear cells which resemble Anitschkow myocytes ($\times 250$).

adequate histologic study of the heart valves. Twelve of the sixteen animals exhibited a slight to moderate focal interstitial myocarditis (Fig. 7). These lesions consisted of dense accumulations of inflammatory cells, predominantly lymphocytes and plasma cells. In one animal (Rabbit 14) there was marked medial necrosis of the aorta which resembles that described by Erdheim²¹ and by Moritz.²² This lesion was seen in the proximal descending aorta and affected the midportion of the media. It involved the entire circumference, and many small cystic areas were seen (Fig. 9). There was no significant inflammatory reaction. The aorta of Rabbit 15 showed a lesser degree of medial necrosis; here the process was focal and appeared more acute. There was fibrinoid necrosis of the collagenic and elastic tissue of the media accompanied by an infiltration of large cells which resembled Anitschkow myocytes (Fig. 10). Sections of aorta were available from only eight of the sixteen animals.

In addition to the vascular lesions, the following pathologic changes warrant special comment.

Kidneys: Five of the six animals which received sulfadiazine showed moderate to marked obstructive nephropathy from sulfadiazine (Fig. 11). Obstruction of the collecting tubules by crystals of the drug had produced marked dilatation of the proximal portion of the nephrons. In addition, the epithelium of the collecting tubules adjacent to the impacted crystals showed rather marked degeneration and necrosis in some areas; in other areas there were foci of marked epithelial hyperplasia. Another striking change observed in the kidneys was a moderate to very marked proliferation of the juxta-glomerular apparatus. This occurred in approximately one third of the animals (Fig. 12). The significance of this finding will be discussed later. Focal acute glomerulitis with occasional synechiae and a rare instance of "crescent" formation were observed in four animals (Rabbits 1, 3, 8, and 9). In two of these animals (Rabbits 1 and 8) there was, within tubules considerable protein precipitate and a few erythrocytes.

Spleen: In addition to a fairly constant follicular hyperplasia, there was proliferation of loose connective tissue about many of the Malpighian arteries appearing as a relatively cell-poor, net-like central area within the corpuscle. The splenic sinuses of many of the animals contained an increased proportion of polymorphonuclear leucocytes, and in some there was considerable blue-staining granular material resembling nuclear debris, much of which was within macrophages. This latter change was seen most frequently in animals which died shortly (one or two days) after injection of horse serum.

Liver: Although the majority of the animals showed a slight to moderate increase in fibrous connective tissue in the portal triads, accompanied by focal collections of inflammatory cells, the significance of these changes was obscured by the parasitic infection present within the group. Occasional areas of recent or healing focal necrosis, not in association with portal triads, were observed in three animals (Rabbits 1, 6, and 9). Hartley and Lushbaugh²³ have reported focal hepatic necrosis in hypersensitive rabbits following injection of specific antigen.



Fig. 11.—Rabbit 14, nineteenth day. Section through the medulla of the kidney showing marked distention of the collecting tubules by sulfadiazine crystals accompanied by focal necrosis and acute interstitial nephritis ($\times 125$)

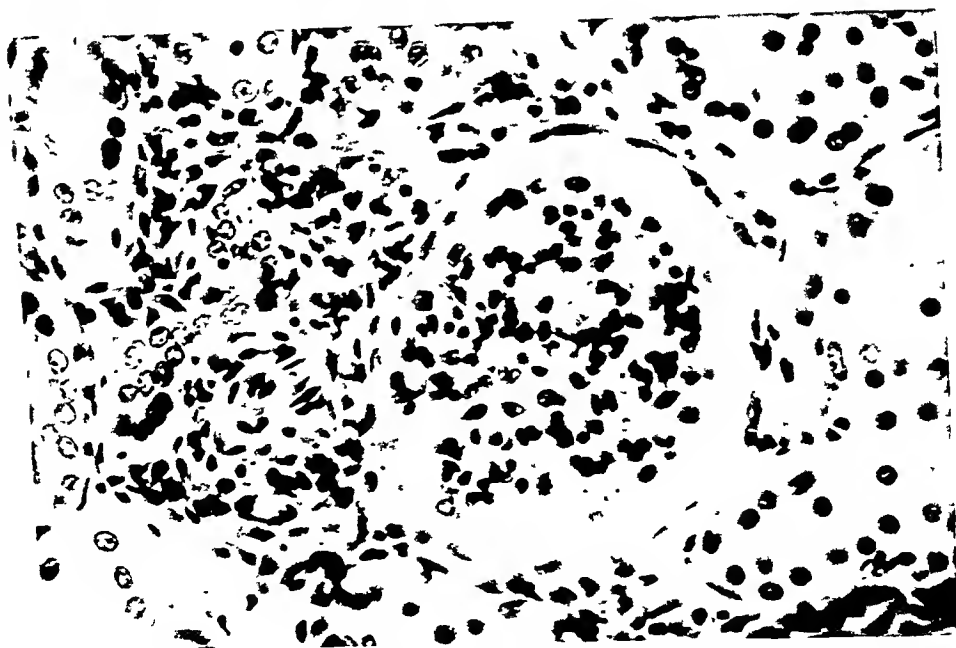


Fig. 12.—Rabbit 2, twenty-sixth day. Section of the cortex of the kidney showing marked proliferation of the juxta-glomerular apparatus ($\times 625$)

Scrous Membranes: Focal fibrous thickening of the peritoneum associated with slight cellular infiltration (lymphocytes and polymorphonuclears) were seen in five animals.

Bone Marrow: Although leucocyte or erythrocyte counts were not determined in this series, a marked hypoplasia of the femoral marrow was observed in one animal (Rabbit 16). Death in this animal apparently resulted from an overwhelming pulmonary infection. In spite of the large numbers of bacteria which were present in the protein-rich edema fluid of the lung, no polymorphonuclear leucocytes were evident. This appearance is quite typical of that observed in human beings dying of agranulocytosis (malignant granulocytopenia). No polymorphonuclear leucocytes were seen in this very hypoplastic femoral marrow. Hypoplasia of the marrow was encountered in two other animals of the group which had received sulfadiazine and horse serum. Additional experiments to determine more precisely the effect of such treatment upon circulating leucocytes and the bone marrow have been performed and will be reported later.

DISCUSSION

We have demonstrated that large doses of horse serum given intravenously, alone or in combination with orally administered sulfadiazine, will produce arterial lesions in rabbits which appear identical with those of periarteritis nodosa. This confirms the observations of Rich and Gregory.⁴ This fact in itself does not, however, answer the following questions: Is the inflammatory response due to a focal "hypersensitive" reaction? If so, why does it localize in small and medium-sized arteries rather than in those organs which are thought to form antibody and which presumably contain antibody in greater concentration (spleen, lymph nodes, liver, bone marrow)? If, on the other hand, these arterial lesions are due to the intravascular action of a toxic antigen-antibody complex, why are not the most marked lesions produced where the circulation is slowest and the vessels most delicate (veins and capillaries)?

The fact that hypersensitivity is associated with a wide variety of inflammatory lesions has been well established experimentally. The most familiar of these are: (1) the Arthus phenomenon, which can vary from a focus of slight erythema to a massive hemorrhagic and necrotizing lesion, and (2) the Anser phenomenon, in which localization of the allergic reaction can be brought about independently of any local instillation of antigen or antibody and in which the lesion may be principally proliferative in certain stages. In experimental animals, glomerulonephritis, a proliferative inflammatory response, can be produced by allergic reaction using nephrotoxin (antibody specific for renal tissue). From a clinical aspect, the direct relationship of serum sickness, asthma, contact dermatitis to the hypersensitive state has long been evident. With reference to the specific problem of periarteritis nodosa, however, one might ask why this disease, if it has an allergic basis, does not occur more often following such routine clinical procedures as passive sensitization with antitetanic horse serum. We are inclined to agree with Rich and Gregory that it is probably a matter of dosage. In this laboratory we had not previously observed vascular phenomena of the sort described here, although in

numerous instances rabbits had been injected repeatedly with various antigens including horse serum; never before, however, had such large doses of antigen been given. The type of antigen employed is unquestionably important also. Horse serum, with its relatively large molecular components, has been shown to persist in the circulation of a normal rabbit for fifteen days or longer.^{24, 25} Its disappearance at this time coincides with the development of active immunity.

Ante-mortem observations made during the course of our experiments demonstrate that circulating antibodies to the horse serum were present while the vascular lesions were developing. A state of hypersensitivity was demonstrated also by the positive Arthus and ear reactions, and the elevations of temperature observed. The lack of quantitative correlation between these reactions is probably dependent upon the fact that horse serum contains multiple antigens. Thus, it is possible that the various manifestations may have been related to different antigen-antibody reactions. For example, one component of the serum might have been principally responsible for the temperature response and another for the ear reaction. In this regard, it is of interest that Jones and Fleisher²⁶ found that the pseudoglobulin portion of horse serum contained the substance responsible for "serum sickness" in rabbits. It is pertinent also that various procedures used commercially in the treatment of immune serums effect a marked reduction in the "serum sickness factor" with but little apparent effect on the antibody fraction. Further experiments are indicated to determine whether single purified proteins in large doses can produce an arteritis and periarteritis. Under the conditions of such an experiment, quantitative correlation among the various immune and allergic reactions might well be apparent.

Concerning the vascular localization of these inflammatory lesions, we originally considered that there might have been an initial injury to small arteries and arterioles resulting from the sudden infusion of a large amount of serum (an amount equal to approximately one-seventh the total blood volume). A necrotizing antigen-antibody reaction localizing at such a site of injury would be somewhat comparable to that illustrated by the Auer phenomenon.²⁷ Accordingly, the rate of the initial injection of horse serum was widely varied in our rabbits in order to produce differing degrees of vascular "overloading." It was found that the rate of infusion (whether two and one-half minutes or over a period of eight hours) bore no relation to the severity of the arterial lesions that resulted.

Since a characteristic response to anaphylaxis in rabbits is spasm of small arteries and arterioles,²⁸⁻³⁰ it is possible that the combination of circulating antigen with antibody, as it is slowly formed and liberated, might lead to a persistent diffuse or focal arterial and arteriolar spasm. Certainly the aural changes described by Fleisher and Jones,¹⁵ as characteristic of serum sickness in rabbits, indicate a marked vascular response. The fact that Rich and Gregory lost three of fourteen animals from anaphylactic shock at the time of the second and third injections of horse serum demonstrates that this method of sensitization can prepare rabbits for this type of allergic reaction.

In our series of sixteen rabbits, none died of anaphylactic shock, although three exhibited moderate dyspnea after the second injection.

The observations¹⁰⁻¹⁴ that vascular lesions, similar in many respects to periarteritis nodosa, can follow experimentally induced renal hypertension in rats, rabbits, and dogs contributes additional evidence that vascular spasm may provide a *locus minoris resistentiae* in effecting the localization of inflammatory reaction to small arteries. Wilson and Byron¹¹ have suggested that marked arteriolar constriction and the accompanying hypertension act together to produce arterial necrosis in hypertensive animals. Others stress the fact that in the majority of instances in which necrotizing and proliferative arteritis complicates experimentally induced hypertension, there has been an associated infectious process.^{12, 13, 31} It is altogether possible that such infectious lesions provide the stimulus for hypersensitivity which, in conjunction with arteriolar spasm, effects an allergic arteritis. It may be that the very marked hyperplasia of the juxta-glomerular apparatus which was observed in a number of our animals was evidence of hypertension. Hyperplasia of the juxta-glomerular apparatus, similar to that observed here, is frequently seen in human cases of hypertension and is an almost constant feature of some types of experimental hypertension. It has been suggested that in such instances this myoneural apparatus causes a persistent spasm of the afferent glomerular arterioles and produces renal ischemia which is in turn responsible for hypertension. We are inclined to believe that arterial injury from prolonged constriction or spasm may provide a localizing mechanism which contributes to the development of periarteritis nodosa. The frequent occurrence of hypertension in periarteritis nodosa is well known. Of 177 cases of human periarteritis nodosa reviewed by Logue and Mullins,³² 53 per cent exhibited hypertension. Of the eleven cases which they themselves carefully studied, they state, "Hypertension was present in every patient at some time during the course of the disease." Studies are in progress to determine whether or not experimentally induced periarteritis nodosa is accompanied by hypertension and, if so, whether the hypertension exerts some causal influence or is simply an effect.

The mechanism previously described would seem to explain the marked similarity between those vascular lesions resulting from experimental hypertension and those observed in rabbits following large doses of a foreign serum. The medial necrosis observed in the aortas of Rabbits 9 and 15 might be explained on a similar basis except that here the arterioles of the vasa vasorum were involved.

Our observations differ in several respects from those of Rich and Gregory. They found that animals which received both horse serum and sulfadiazine exhibited widespread necrotizing and inflammatory arterial lesions of essentially the same type and degree as did those animals which received only horse serum. In our series this was not the case (Table II). However, Rich and Gregory found that the daily administration of 0.5 Gm. per kilogram of sulfadiazine was not toxic to rabbits; whereas five of the six rabbits in our series which received this dosage died before the end of the experiment, and at autopsy they presented a moderate to marked obstructive nephropathy

due to the presence of sulfadiazine crystals in the collecting tubules. It is possible that this toxic reaction may have so injured the affected animals that their ability to produce antihodies was considerably depressed, leading in turn to diminished allergic response and the less severe arterial lesions which occurred. The vascular lesions observed by us were qualitatively the same as those described by Rich and Gregory, but even in the animals receiving horse serum alone they were not as numerous nor were they so generalized. Correspondingly, the febrile reactions and erythema of the ears which we observed appear to have been somewhat milder. Careful histopathologic examination did not reveal the acute diffuse glomerulonephritis which Rich and Gregory observed in ten of their fourteen animals. In four of our rabbits there was microscopic evidence of focal glomerulitis, with an occasional synechia and a rare instance of crescent formation. In two of these animals erythrocytes and hyaline casts were seen in the tubules. However, the focal nature and mildness of these lesions were remote from the typical picture of glomerulonephritis in human beings.

SUMMARY AND CONCLUSIONS

In an attempt to produce experimental periarthritis nodosa, a group of sixteen white male adult rabbits was injected intravenously with large doses of sterile normal horse serum. The rate at which this first injection was given was intentionally varied in an attempt to produce differing degrees of vascular "overloading." At certain intervals following this first injection, intravenous horse serum was again administered to most of the animals. During the experimental period six of the rabbits received two nine day "courses" of sulfadiazine by mouth; however, five of them failed to survive the second period of treatment. Rectal temperatures were determined daily for each animal from the first to the twenty-sixth day. At appropriate intervals their ears were examined for erythema and edema of the type described by Fleisher and Jones. Cutaneous hypersensitivity to horse serum and sulfadiazine was determined, and precipitin antibodies to horse serum (and sulfadiazine) were titrated. At varying times, from the seventeenth to the thirty-fourth day, the rabbits were autopsied and representative tissues taken for microscopic study.

1. The majority of the rabbits exhibited definite but varying degrees of "serum sickness," cutaneous hypersensitivity, and precipitin formation following the administration of horse serum. In general, there was poor quantitative correlation among the results of these ante-mortem observations and the degree and extent of arteritis observed post mortem. The relationship of the multiple antigenic proteins in horse serum to this lack of correlation is discussed.

2. Arterial lesions, varying from a generalized acute necrotizing arteritis and periarthritis to a less spectacular and more focal subacute and proliferative arteritis and periarthritis, were observed in the majority of the animals. These latter lesions especially were seen most frequently in the heart. On the whole, the vascular lesions observed were qualitatively similar to those described by Rich and Gregory but quantitatively less marked and less generalized.

3. As a rule, the most marked arterial lesions were observed in animals which had shown the greatest elevations in temperature. The most marked arteritis occurred in rabbits killed on the nineteenth and twenty-sixth days. There was no correlation between the rate at which the initial injection of horse serum was given and the intensity or frequency of the resulting arterial lesions.

4. As a group, the animals receiving sulfadiazine in addition to the horse serum produced less precipitin antibody and manifested a lower degree of temperature response and cutaneous sensitivity than did those animals which received horse serum alone. They also exhibited much less arteritis histologically. This may have been related to the toxic obstructive nephropathy which occurred in five of the six animals receiving sulfadiazine.

5. Attempts to demonstrate cutaneous hypersensitivity or circulating antibodies to sulfadiazine resulted in questionable or negative reactions.

6. A diffuse erythematous eruption in the skin of the abdomens of four rabbits was observed following the second large dose of horse serum. Histopathologic changes, as well as the time of their occurrence, suggest that they were cutaneous manifestations of "accelerated serum sickness."

7. Lesions resembling Aschoff bodies were observed occasionally in the myocardium, and subacute focal interstitial myocarditis was common. The aortas of two rabbits showed a cystic medial necrosis.

8. In addition to the obstructive nephropathy, there was a marked hyperplasia of the juxta-glomerular apparatus in the kidneys of approximately one third of the animals. The possible significance of this lesion is discussed. A slight focal glomerulitis was observed in four animals.

9. Toxic or allergic damage to the bone marrow is suggested by the fact that one animal developed agranulocytosis and two others exhibited rather marked hypoplasia of the bone marrow.

10. It is suggested that in the arteritis and periarteritis which follows large doses of horse serum, prolonged spasms of the small and medium-sized arteries may provide the *locus minoris resistentiae* necessary to localize this allergic inflammatory reaction. The possible pathogenic relationship of such allergic arteritis and periarteritis to similar lesions observed in experimental hypertension is discussed.

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PARACOLON BACILLI AND THEIR RELATION TO URINARY TRACT INFECTIONS

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PARACOLON bacilli, commonly regarded as aberrant or variant forms of the typical coliform bacilli, are generally described simply as late-lactose-fermenting strains of *Escherichia* and *Aerobacter*. This is, indeed, their chief common characteristic, and although they may differ from typical coliform bacilli in other cultural reactions, they are most noted for their failure to ferment lactose promptly with the production of marked acid and gas as do typical strains of *Escherichia* and *Aerobacter*.

This group of gram-negative bacilli has recently attracted considerable attention in certain fields of bacteriologic investigation. They have been studied by the sanitary bacteriologists to whom they are of interest as a possible index of the fecal pollution of water whose presence is not detected by the accepted methods of water analysis based on the prompt fermentation of lactose with acid and gas.¹⁻³ They have also been the subject of extensive investigation by the public health laboratories engaged in the study of enteric infections and outbreaks of acute gastroenteritis.⁴⁻⁷ Here this group of gram-negative bacilli are of interest not so much as possible etiologic agents in intestinal disease, although the possibility of their significance in gastroenteritis has frequently been suggested,^{1, 3, 4, 6, 7} but rather because their close cultural resemblance to the true enteric pathogens (*Eberthella*, *Salmonella*, and *Shigella*) often makes their differentiation a problem.^{3-5, 7-12} Frequently they are falsely identified as one of the pathogenic, intestinal gram-negative bacilli, thus leading to misinterpretation of the significance of their presence in stool specimens from patients and carriers. This error these public health laboratories are striving to eliminate through a more thorough study and knowledge of the characteristics of these organisms. Last, certain of the paracolon bacilli have been used for genetic studies as organisms which readily lend themselves to the investigation of bacterial variation.^{10, 11, 13}

With the exception of these fields, very little attention has been directed to the paracolon bacilli. Their occurrence in clinical material other than stool specimens has not been reported, with the exception of a few instances of their isolation from urine cultures, as will be reviewed later in this paper. Accurate information concerning them is not widely disseminated, and complete descriptions are to be found only in a few reports in the literature^{1-4, 8} Textbooks of medical bacteriology contain at best only brief references to these organisms, and these descriptions are too inadequate to permit recognition of the various types of paracolon bacilli on this basis. The difficulty in identifying many of

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the paracolon bacilli, and the fact that the knowledge of and interest in these organisms appears to be confined to a few fields, suggests the possibility that their identity as such is not recognized by many and therefore the frequency of their occurrence is unknown. In any case, it is apparent that the relation of paracolon bacilli to infections other than those of the intestinal tract deserves investigation.

The isolation of a considerable number of paracolon bacilli from urine specimens has prompted the investigation of these organisms along several lines. We have been interested, first, in the cultural characteristics of paracolon bacilli and methods for their identification; second, in their significance in the urinary tract; and, third, in their relation to typical coliform bacilli from the same source. This report will concern itself with these three phases of the problem.

DESCRIPTION OF PARACOLON BACILLI

Excellent descriptions of the cultural characteristics of the various types of paracolon bacilli are to be found in the recent literature. Perhaps the best are those of Stuart and his co-workers. In 1940, Stuart, Mickle, and Borman³ described the variations in lactose fermentation. In 1943, Stuart, Wheeler, Rustigian, and Zimmerman⁴ elaborated the earlier description and reported the characteristics of paracolon bacilli as of the *Escherichia*, *Aerobacter*, and intermediate groups. In 1944, Borman, Stuart, and Wheeler⁵ suggested the establishment of a new genus, the *Paracolobactrum*, to include these organisms and proposed the following three species: *Paracolobactrum aerogenoides*, for those related to the *Aerobacter* genus; *Paracolobactrum intermedium*, for those resembling the intermediate coliform bacilli; and *Paracolobactrum coliforme*, for those related to the typical *Escherichia*. Additional information concerning these organisms may be found in the publications of Parr and associates.^{1, 2, 9-11}

TABLE I. CULTURAL CHARACTERISTICS OF TYPICAL COLIFORM BACILLI

GENUS	MOTILITY	LACTOSE	DEXTROSE	SUCROSE	MANNITOL	INDOL	MALONATE OR CITRATE
<i>Escherichia</i>	+/-	AG 24 hr.	AG	AG/-	AG	+	-
<i>Aerobacter</i>	-	AG 24 hr.	AG	AG	AG	-	+
Intermediates	+/-	AG 24 hr.	AG	AG/-	AG	+/-	+/-

AG, Acid and gas.

+/-, Positive or negative.

It is not the purpose of this report to review in detail all the accumulated knowledge concerning the paracolon bacilli but rather to give as simply as possible an adequate description of these organisms, particularly as they occur in urine cultures. It is difficult to give a good, concise description of the paracolon bacilli because of the multiplicity of cultural types which may be encountered. According to various authors, they may arise from any of the coliform bacilli, that is, from *Escherichia*, *Aerobacter*, or the intermediate group.^{1, 3-5, 8, 9} The cultural characteristics of these typical coliform bacilli are given in Table I.

As a result of this proposed difference in origin, the paracolon bacilli will differ in their cultural reactions as do the groups from which they arise. This alone produces a number of cultural types. But the paracolon bacilli are characterized by their variation from these typical coliform bacilli. In the first place, they vary considerably in their fermentation of lactose. Typical coliform bacilli, that is, *Escherichia*, *Aerobacter*, and the intermediate types, ferment lactose promptly, within twenty-four hours, with the production of acid and marked gas (20 per cent or more). The aberrant coliform bacilli, as the paracolon bacilli are frequently called, may have any of the following atypical reactions in lactose¹⁻⁴:

1. Ferment lactose slowly (forty-eight hours and over) with acid and gas.
2. Ferment lactose slowly with acid and slight gas. Gas production may vary from a bubble to 10 per cent.
3. Ferment lactose slowly with acid and no gas.
4. Ferment lactose slowly and atypically at 37° C. but give a typical acid-plus gas reaction at 20° C. These organisms have been isolated only from water, milk, and soil and not from human material.³
5. Fail completely to ferment lactose.

It is these differences in lactose fermentation which are generally considered the chief characteristic of the paracolon bacilli. However, it must be understood that these organisms may differ in a number of characteristics other than lactose fermentation, as has been repeatedly pointed out by investigators.^{1, 2, 4, 9, 11, 13} They may fail to ferment or may ferment atypically, that is, slowly, with slight gas production or with no gas carbohydrates other than lactose, the most important of these being dextrose, sucrase, and mannitol. They may lack the capacity to produce indol, and other characteristic reactions may also be absent. Lewis¹³ stresses the fact that one strain may be unstable for one test substance, for example, lactose, or for half a dozen; thus "a family tree of astounding complexity" may be developed.

In our laboratory we have used the following characteristics in the study of the paracolon bacilli: motility; growth on desoxycholate agar plates; the fermentation of lactose, dextrose, sucrase, and mannitol; the production of indol; and the utilization of sodium malonate as the sole source of carbon. The malonate broth of Leifson¹⁴ has been used in place of citrate agar employed in many laboratories and has been found to be very satisfactory in differentiating *Escherichia*, which fail to utilize sodium malonate, from *Aerobacter*, which give a positive reaction. The fermentation of carbohydrates other than those listed has been investigated in various instances but has not proved helpful in the identification of these organisms. Agglutination tests with specific antiserum have been done in all cases where cultural similarity made the differentiation of the paracolon bacilli from the enteric pathogens difficult.

Using these characteristics, the paracolon bacilli which we have encountered in urine cultures have fallen into certain definite cultural types. In all instances the colonies on desoxycholate agar plates were colorless, indicating failure to ferment lactose promptly. On blood agar and on desoxycholate agar

plates, the colonies were usually considerably smaller than those of the typical coliform bacilli and occasionally were so atypical as to resemble those of streptococci. Of the other characteristics studied, we have found the most common variations to be as follows:

1. Failure to ferment lactose promptly; acid and gas produced after forty-eight hours to ten days incubation.
2. Delayed fermentation of lactose with acid and little or no gas.
3. Fermentation of dextrose with acid and no gas.
4. Failure to ferment sucrose.
5. Fermentation of sucrose with acid and no gas.
6. Failure to ferment mannitol.
7. Fermentation of mannitol with acid and no gas.
8. Failure to produce indol.
9. Loss of motility.

These differences define the cultural types listed in Table II.

One specific type of paracolon bacillus deserves special mention since its outstanding characteristic is not shown in Table II. This is the organism known as *Bacterium coli-mutabile* and originally described by Neisser¹⁵ and Massini.¹⁶ The recent literature contains excellent descriptions of this organism.^{1, 3, 10, 13} In lactose fermentation tubes *Bact. coli-mutabile* produces acid and a small amount of gas after forty-eight hours incubation, gas production increasing with prolonged incubation. Colonies on desoxycholate agar plates are colorless after forty-eight hours incubation. In three to five days, however, small, pink, secondary colonies, or papillae, appear growing on or out of the original colorless colonies. Subcultures from the basic, colorless colony ferment lactose slowly, while those from the colored, secondary colonies ferment lactose in twenty-four hours with acid and gas as do the typical coliform bacilli. These unstable strains may arise from organisms of the *Escherichia*, *Aerobacter*, or intermediate group. Stuart, Mickle and Borman³ object to the name "*coli-mutabile*," since it suggests that these bacilli are all related to the *Escherichia*; they propose the term "*papillae-forming coliforms*" to describe these organisms. Although they occur infrequently and none are included in the series to be reported, we have isolated a number of this type of paracolon bacilli from urine cultures, and therefore their description has been included here.

As has been pointed out in Table II, the paracolon bacilli may culturally resemble *Salmonella*, *Proteus*, *Shigella*, or *Eberthella*. This resemblance is repeatedly stressed in the literature.^{3-5, 7-12} In most instances, however, well-defined differences do exist which make cultural differentiation possible. The paracolon bacilli most frequently encountered in urine cultures are the late-lactose-fermenting varieties which produce acid and gas in the fermentation of carbohydrates. These may superficially resemble *Salmonella* or *Proteus*, although many of them are nonmotile. The fermentation of mannitol will eliminate *Proteus*, while a spreading growth on infusion agar will obviously confirm the identification. *Salmonella* and *Eberthella* will be eliminated by the production of indol or the utilization of sodium malonate. In the case of strains

TABLE II. CULTURAL CHARACTERISTICS OF PARACOLON BACILLI MOST FREQUENTLY ISOLATED FROM URINE CULTURES

LACTOSE	DEXTRIOSE	SUCROSE	MANNITOL	INDOL	MALONATE OR CITRATE		MOBILITY	CULTURALLY RESEMBLES	AGGLUTINATION* WITH SPECIFIC ANTISERUM
AG	AG	AG/-	AG	+	-	-	+/-	Salmonella or Proteus	Negative
48 hr. to 10 days AG	AG	AG	AG	-	+	-	-	Salmonella or Proteus	Negative
48 hr. to 10 days Negative	AG	AG/-	AG	+	-	-	+/-	Salmonella or Proteus	Negative
Negative	AG	AG	AG	-	+	-	-	Salmonella or Proteus	Negative
Acid only	A	A/-	A only	-	-	-	-	Shigella dispar	Negative
48 hr. to 10 days Acid only	A only	A/-	A only	-	-	-	-	Shigella sonnei	Negative
48 hr. to 10 days Negative	A only	-	A only	-	-	-	+/-	Shigella paradysenteriae Eberthella typhosa	Negative
Negative	A	-	-	+	-	-	-	Shigella ambigua Shigella dysenteriae	Negative
Negative	A only	-	-	-	-	-	-		Negative

AG, Acid and gas.

A, Acid.

+/-, Positive or negative.

*Antiserum to be used will be that suggested by cultural similarity as indicated in Column 8. For example, a paracolon bacillus culturally resembling *Shigella dysenteriae* should be tested with *Sh. dysenteriae* antiserum.

resembling the *Shigella*, however, the complete cultural similarity often makes it necessary to set up agglutination tests with specific antiserum as indicated by the cultural reactions. If no agglutination occurs, the identity of the organism as a paracolon bacillus is strongly suggested.

The ease with which the paracolon bacilli may be confused with the true enteric pathogens cannot be overemphasized. Frequently they are erroneously classified as a member of the *Salmonella* or *Shigella* genera, although the resemblance is not usually sufficiently close to allow actual species identification. An illustration of the confusion which may result from the failure to recognize an organism as a paracolon bacillus can be found in the culture reports included in the history of a single patient on the gynecological service of the Johns Hopkins Hospital. This is the case of a woman with bilateral ureteral strictures and chronic pyelitis who has had fifty-two hospital admissions for cystoscopic treatments. For fourteen years all urine cultures were positive for *Escherichia coli*. During the succeeding ten years the urine culture reports in chronological order were as follows:

1. Unidentified gram-negative rods, lactose negative; dextrose, sucrose, and mannite fermented with acid and gas, gelatin negative, motility negative.
2. Nonmotile gram-negative rods with the sugars of *B. enteritidis*.
3. *B. coli*.
4. Nonmotile gram-negative rod, sugars of *B. enteritidis*.
5. *B. coli*.
6. Nonmotile gram-negative rod, sugars of *Salmonella enteritidis*.
7. Nonmotile gram-negative rod, acid and gas in dextrose, sucrose, and mannite, probably slow lactose fermenter.
8. *B. alkaligenes*.
9. Gram-negative rods, culturally *Proteus* (probably atypical strain).
10. *B. proteus*, probably atypical strain.
11. *B. proteus*, probable atypical strain.
12. Gram-negative rods, ferments the usual carbohydrates with acid and gas but does not ferment lactose (after eight days). Gelatin not liquefied.
13. Gram-negative rods which ferment dextrose and sucrose with acid and gas and do not ferment lactose. Do not liquefy gelatin.
14. Paracolon bacilli.
15. *E. coli* (in five succeeding cultures).
16. Atypical *Proteus*.
17. *Salmonella enteritidis*.
18. Paracolon bacilli (in six succeeding cultures).

These urine cultures were done by a number of bacteriologists in several laboratories over a period of twenty-four years. The last six cultures, all identified as paracolon bacilli, were studied in our laboratory and were definitely proved to be paracolon bacilli related to *Escherichia*. We do not feel it unreasonable to assume that the many unidentified or atypical organisms reported in previous years were also paracolon bacilli; indeed, the cultural reactions when included in the bacteriologic report confirm this point of view. In the light

of our experience with the variation of *Escherichia* and *Aerobacter* to paracolon bacilli in the urinary tract, as will be reported later, the assumption that these organisms were paracolon bacilli seems logical. If this assumption may be made, then certainly this case vividly illustrates the need for greater knowledge of the paracolon bacilli in medical bacteriology.

How may these errors in identification and the confusion of paracolon bacilli with *Salmonella*, *Eberthella*, *Shigella*, or *Proteus* be avoided? Knowledge of the various cultural characteristics of these aberrant coliform bacilli is, of course, essential, and a careful cultural and serologic study of the organisms will usually permit satisfactory differentiation. As far as the identification of paracolon bacilli from urine cultures is concerned, we have found certain points helpful in suggesting the identity of these organisms. A hint as to their possible identity may be gained from the fact that in these cases there is no clinical evidence to suggest infection with one of the *Salmonella*, *Eberthella*, or *Shigella*. Also, as will be discussed later in this report, paracolon bacilli most frequently occur in urine specimens together with typical coliform bacilli or from patients whose preceding urine cultures yielded *Escherichia* or *Aerobacter*. The finding of a lactose-negative, gram-negative bacillus under these circumstances is highly suggestive of a paracolon bacillus.

RELATION OF PARACOLON BACILLI TO URINARY TRACT INFECTIONS

A review of the literature on paracolon bacilli and on urinary tract infections has not yielded a great deal of information concerning the occurrence and significance of these organisms in urinary tract disease. Parr¹ states that "although practically all of the atypical coliform bacilli were first isolated from feces, they were early found in the urine." There he is referring to reports of Mair¹⁷ and Wilson.¹⁸ In 1906, Mair described organisms from two cases of cystitis which "agree with *B. coli*" with the exception of the fact that they produce no gas in lactose and glucose. These organisms Mair described as "paracolon bacilli." In 1908, Wilson reported six strains of gram-negative bacilli from urinary tract infections which resembled colon bacilli but differed in their failure to produce gas in the fermentation of carbohydrates. The classical work on the subject is that of Dudgeon and his co-workers in 1922 to 1927.^{7, 19, 20} They report a series of acute urinary tract infections in which the etiologic agent was a "slow-lactose fermenting *B. coli*." Since then writers on the subject of paracolon bacilli frequently mention in passing that these organisms may be responsible for infections of the urinary tract, referring to Dudgeon's work as authority for such statements and seemingly regarding the relationship as a probability rather than an established fact.^{1-3, 12, 21} As far as actual isolation from urine cultures is concerned, only two recently reported instances could be found. Kennedy, Cummings, and Morrow¹² describe twenty-two strains of paracolon bacilli, four of which were isolated from urine. Cross²² reports a "bacillus para coli" in a series of twenty-five organisms isolated from urinary tract infections. In a fairly comprehensive review of the recent literature on infections of the urinary tract made in connection with this and another report,²³ no other mention of paracolon bacilli in urine cultures could be found.

In the literature on urinary tract infections there is to be found, however, numerous reference to organisms described as unclassified gram-negative bacilli or as unidentifiable *Salmonella*, *Eberthella*, or *Shigella* which our experience suggests may well have been paracolon bacilli. For example, Marple²⁴ reports the isolation from urine from a case of chronic cystitis of a "gram-negative bacillus giving the sugar reactions of paratyphoid group but serologically unidentifiable." Cerf and Goldman²⁵ describe "gram-negative bacilli which do not show the sugar fermentations of known pathogens," and Stalker and Schulte²⁶ report the isolation from urine of four strains of "*Shigella* (species?)."

Hill, Seidman, Stadnichenko, and Ellis,²⁷ in their excellent study of two hundred gram-negative bacilli isolated from cases of genitourinary infection, report "sixteen miscellaneous cultures." These were identified as one *Alkaligenes*, five strains of *Eberthella*, nine *Shigella*, and one *Salmonella*. Of the fourteen *Eberthella* and *Shigella* strains one was not classified and the others were tentatively assigned to certain obscure species, although they did not correspond in all respects to recorded descriptions of these species. The bacillus considered *Salmonella paratyphi* fermented sucrose but in spite of this discrepancy was so classified because it was agglutinated by *S. paratyphi* immune serum. Since no recognized paracolon bacilli were included in this large series of 200 gram-negative bacilli and since, as will be discussed later, we have encountered these organisms with some frequency in urine specimens, it seems logical to presume that a number of these miscellaneous bacilli were paracolon bacilli of the type culturally resembling but not identical with certain *Eberthella*, *Shigella*, and *Salmonella*. The fact that the one sucrose-positive strain which was identified as *S. paratyphi* agglutinated with *S. paratyphi* antiserum does not eliminate the possibility of its being a paracolon bacillus, since Lommel,²⁸ using malachite green and safranin, suppressed lactose fermentation in cultures of colon bacilli and reports that three such experimentally produced lactose-negative strains became agglutinable with *Bacterium paratyphosum* B. antiserum.

Levine²⁹ has published a report on "Unclassifiable Gram-negative Rods in Urinary Infections." These organisms are described as failing to correspond to species described in Bergey's *Manual of Determinative Bacteriology* or as serologically differing from known species. Among the bacilli included in this report are strains which resemble *Esch. coli* but do not ferment lactose. Others resemble *Shigella* but do not agglutinate with known antiserum. Also described are "strains which are related to the *Salmonella* group biochemically but either form indol or are serologically distinct from the recognized *Salmonella*." In another report Levine³⁰ again refers to unclassifiable gram-negative bacilli, describes strains similar to those referred to previously, and includes a chart giving the cultural reactions of three types of atypical bacilli. The characteristics of a number of the organisms included in these publications strongly suggest that they may be paracolon bacilli; their description coincides with that of organisms of this group which we have isolated from urine specimens, and their identity as aberrant forms of coliform bacilli seems highly probable.

If such be the case and if a number of the unidentified bacilli reported from urinary infections are paracolon bacilli, then certainly attention need be directed to the characteristics of paracolon bacilli occurring in urine cultures, so that they may be recognized and correctly identified.

As has been stated, we have isolated a number of paracolon bacilli from urine cultures. The specimens from which these organisms were cultured were received from women patients on the gynecological and obstetrical services of the Johns Hopkins Hospital. The organisms were isolated by our routine procedure as described in Schaub and Foley.³¹ They produced colorless colonies on desoxycholate agar, indicating their failure to ferment lactose promptly and were isolated from such colonies, and their characteristics as tabulated in Table II were studied. Agglutination tests with appropriate antiserum as indicated by the cultural reactions have been carried out in all instances where cultural reactions alone were insufficient for differentiation.

A study of the incidence and significance of these paracolon bacilli in the female urinary tract has been made. The series includes all cases from both the gynecological and obstetrical services, for the year 1945, in which these organisms were found. The incidence of paracolon bacilli in this series is given in Table III. As shown here, 100 strains of paracolon bacilli were isolated, eighty-six from patients on the gynecological service and fourteen from obstetrical cases. These represent 3.02 per cent of the total number of urine cultures received in the laboratory during this year and 4.3 per cent of the positive cultures obtained.

TABLE III. OCCURRENCE OF PARACOLON BACILLI IN URINE SPECIMENS FROM WOMEN PATIENTS ON GYNECOLOGICAL AND OBSTETRICAL SERVICES

SERVICE	TOTAL NUMBER OF URINE SPECIMENS	NUMBER OF POSITIVE CULTURES	NUMBER OF PARACOLON BACILLI	PERCENTAGE OF TOTAL URINE CULTURES	PERCENTAGE OF POSITIVE CULTURES	NUMBER OF PATIENTS
Gynecology	2,486	1,847	86	3.06	4.6	46
Obstetrics	822	452	14	1.7	3.09	11
	3,308	2,299	100	3.02	4.3	57

The 100 strains of paracolon bacilli were isolated from fifty-seven different patients as shown in Table III. Histories were available for fifty-one of these patients, and these histories were carefully reviewed for evidence of urinary tract infection. The results of this study are given in Table IV. Here it is shown that of the forty-patients on the gynecological service eight were without symptoms of urinary tract infection, five with no white blood cells, and three with white blood cells in catheterized urine specimens. Eleven patients showedptoms of cystitis; eight were diagnosed as acute and three as chronic infections. Symptoms of pyelitis were present in twenty-one instances, the infection being acute in thirteen cases, and ten patients having chronic disease. Thus seen that in thirty-five, or 87.5 per cent, of the patients on the gynecological service symptoms of urinary tract infection were present. eleven obstetrical patients six showed no signs or symptoms of urinary tract infection, while the remaining five, or 45 per cent, showed symptoms of acute pyelitis.

TABLE IV. RELATION OF PARACOLON BACILLI TO URINARY TRACT SYMPTOMS

SERVICE	WITHOUT SYMPTOMS		SYMPTOMS OF CYSTITIS			SYMPTOMS OF PYELITIS			TOTAL NUMBER OF CASES
	WITH-OUT WHITE BLOOD CELLS	WITH WHITE BLOOD CELLS	ACUTE	CHRONIC	TOTAL	ACUTE	CHRONIC	TOTAL	
Gynecology	5	3	8	3	11	11	10	21	40
Obstetrics	6	-	-	-	-	5	-	5	11
Total number of cases	11	3	8	3	11	16	10	26	51
Percentage of total number of cases	21.5	5.8	15.6	5.8	21.5	31.3	19.6	50.9	.

The number of chronic infections in which paracolon bacilli were involved deserves comment. Thirteen cases, three of chronic cystitis and ten of chronic pyelitis, a total of 25.5 per cent, are included in this series. This contrasts with the reports of Dudgeon and associates^{19, 20} who emphasize the acute nature of the disease and the rapid, complete recovery of the patients included in the series of cases they reported.

RELATION OF PARACOLON BACILLI TO ESCHERICHIA AND AEROBACTER IN THE URINARY TRACT

We have been impressed with the frequency with which paracolon bacilli either were found following the isolation of *Escherichia* or *Aerobacter* in a previous urine specimen from the same patient, occurred together with normal lactose-fermenting strains of these organisms, or were followed by the isolation of typical coliform bacilli. Histories of the patients from whom paracolon bacilli have been isolated were therefore studied for information concerning the association of the aberrant coliform bacilli with typical *Escherichia* and *Aerobacter*. The results of this study are contained in Table V. Here it is shown that in our series of fifty-one patients for whom histories were available, in twenty-seven, or 52.9 per cent, of the cases the paracolon bacilli either were preceded by, occurred with, or were followed by *Escherichia* or *Aerobacter*. Association with *Escherichia* occurred with far greater frequency, 45.9 per cent, than with *Aerobacter*, 7.8 per cent. This association was frequently very close, since in a number of cases the paracolon bacilli not only were preceded by the isolation of *Escherichia* or *Aerobacter* but also occurred with and were followed by typical coliform organisms. The cases in which this occurred are represented in Table V by the figures in parentheses.

Twenty-four, or 47 per cent, of the cases showed no proved association with *Escherichia* or *Aerobacter*. It should be noted, however, that in twenty of the twenty-four cases with no proved association between the aberrant and typical coliform organisms little opportunity to demonstrate such an association was provided. There was only one recorded urine culture on these twenty patients, and that one culture was the one in which paracolon bacilli occurred in pure culture. Thus, of the thirty-one cases in which more than one culture was

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A study of the incidence and significance of these paracolon bacilli in the female urinary tract has been made. The series includes all cases from both the gynecological and obstetrical services, for the year 1945, in which these organisms were found. The incidence of paracolon bacilli in this series is given in Table III. As shown here, 100 strains of paracolon bacilli were isolated, eighty-six from patients on the gynecological service and fourteen from obstetrical cases. These represent 3.02 per cent of the total number of urine cultures received in the laboratory during this year and 4.3 per cent of the positive cultures obtained.

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taken and an opportunity offered to demonstrate association with *Escherichia* or *Aerobacter*, twenty-seven, or 87.1 per cent of these cases showed this association.

Table V also demonstrates that association of paracolon bacilli with *Escherichia* or *Aerobacter* occurs more frequently in chronic than in acute infections; in 80 per cent of the cases with chronic pyelitis as compared with 68.7 per cent of the cases of acute pyelitis and in 66.6 per cent of the patients with chronic cystitis as compared with 42.5 per cent of the cases of acute cystitis. In patients showing white cells only, the incidence of this association was 33.3 per cent, and in patients with no signs or symptoms of urinary tract infection only, 18.1 per cent. The higher incidence in patients with chronic disease may well be explained by the fact that in such cases it is possible to study the infection over a relatively long period of time and to obtain numerous cultures on such patients. Thus an opportunity is afforded to establish an association between these organisms. In acute cases there is frequently only one specimen taken, and thus there is no evidence that an *Escherichia* or *Aerobacter* isolated from this one culture was followed by a variant form or, if a paracolon bacillus was isolated, that it was preceded or followed by typical coliform bacilli.

DISCUSSION

The incidence and pathogenicity of paracolon bacilli in the female urinary tract has been established by the data presented in Tables III and IV. A possible explanation of the presence of these atypical coliform bacilli is afforded by the evidence of the association of these organisms with *Escherichia* and *Aerobacter* in 52.9 per cent of the cases as shown in Table V. As has been pointed out, if only the cases in which more than one culture had been taken are considered, for only in cases with a number of cultures was ample opportunity to prove this association provided, then in 87.1 per cent of the cases the paracolon bacilli were preceded by, occurred with, or were followed by typical *Escherichia* or *Aerobacter*. This close association suggests that in at least a number of cases the organism initiating the infection was an *Escherichia* or *Aerobacter* and that the paracolon bacilli arose from these typical coliform bacilli through variation in the urinary tract. We were interested in determining, if possible, if this theory were tenable and, if so, what factors or conditions might be responsible for this variation.

A review of the literature has not proved fruitful in suggesting what factors may be involved in the production of paracolon bacilli. That they are aberrant forms of *Escherichia* and *Aerobacter* is well established,^{1-5, 8-13} and the nature of the factors influencing this variation has been the subject of considerable speculation. Kline³² suggests that paracolon bacilli are coliform organisms which may have become modified through the influence of unfavorable environment. A number of investigators^{1, 3, 4} have pointed out that the incidence of paracolon bacilli in feces from patients with gastroenteritis is considerably higher than normal. Stuart, Mickle, and Borman³ express the opinion that the presence of the aberrant coliform organisms in such stool cultures "may

TABLE V. ASSOCIATION OF PARACOLON BACILLI WITH TYPICAL STRAINS OF ESCHERICHIA AND AEROBACTER

PARACOLON BACILLI ASSOCIATED WITH	RELATION OF PARACOLON BACILLI TO ESCHERICHIA OR AEROBACTER	NO SIGNS OR SYMPTOMS OF U.T.I.	ISOLATED FROM PATIENTS WITH					NUMBER OF STRAINS	PERCENTAGE OF TOTAL NUMBER OF STRAINS
			WHITE CELLS ONLY	ACUTE CYSTITIS	CHRONIC CYSTITIS	ACUTE PYELITIS	CHRONIC PYELITIS		
Escherichia	Preceded by	1	-	2	2	5	5	15	29.4
	Occurred with	1	1	-	(1)*	2	2	6	11.7
	Followed by	-	-	-	(2)	2	(2)	2	
	Total	2	1	2	2	9	(5)	2	3.9
Aerobacter	Preceded by	-	-	1	-	2	1	23	45.9
	Occurred with	-	-	-	-	(2)	(1)	4	7.8
	Followed by	-	-	-	-	-	(1)	-	
	Total	0	0	1	0	2	-	-	
Number of strains associated with Escherichia and Aerobacter	Preceded by	2	1	3	2	11	8	27	52.9
	Occurred with	-	-	-	-	-	-	-	
	Followed by	-	-	-	-	-	-	-	
	Total	2	1	3	2	11	8	27	52.9
Number of strains with no proved association with Escherichia or Aerobacter	Preceded by	9	2	5	1	5	2	24	47
	Occurred with	-	-	-	-	-	-	-	
	Followed by	-	-	-	-	-	-	-	
	Total	9	2	5	1	5	2	24	47
Total number of strains		11	3	8	3	16	10	51	
Percentage associated with Escherichia or Aerobacter		18.1	33.3	42.5	66.6	63.7	80	52.9	

*Figures in parentheses represent cases included in figures directly above.

*Figures in parentheses represent cases included in figures directly above.

In our series of cases not only did *Escherichia* and *Aerobacter* precede and occur with paracolon bacilli, but in ten instances *Esch. coli* was found following the isolation of paracolon bacilli in pure culture in a previous specimen. This suggests that the aberrant strains may revert in the urinary tract to typical, prompt-lactose-fermenting varieties. This reversion is well known in *Bact. coli-mutabile*^{1-4, 10, 11, 13} in which secondary colonies of lactose-fermenting strains are produced invariably by colonies of the slow-lactose-fermenting form. Investigators have been able to bring about this reversion in other paracolon bacilli,^{3, 5, 12, 13, 37} and we have been able to accomplish this in a number of instances. Therefore, it seems probable that in the urinary tract not only may the aberrant paracolon bacilli be produced from *Escherichia* and *Aerobacter* but also that these paracolon bacilli may, in turn, give rise to typical coliform organisms. Indeed, in eight instances in our series the occurrence of paracolon bacilli in pure culture was both preceded and followed by the isolation of *Escherichia*.

Although we believe that our evidence suggests that the origin of paracolon bacilli in the urinary tract may, in some cases at least, be by way of variation from typical coliform bacilli, our study has yielded no evidence of the factors concerned in this variation. It seemed possible that chemotherapy might play a role in bringing about this change. The presence in the urine of high levels of sulfonamides or penicillin in patients being treated with these substances might possibly be a factor favoring variation. A study of the histories of the patients included in this series did not confirm this theory. In the fifty-one cases reviewed, paracolon bacilli were isolated before the initiation of chemotherapy in forty-six instances. In only five cases did these organisms appear after treatment, once each after the administration of sulfadiazine, sulfasuccidine, and penicillin and in two patients following sulfamerazine therapy. These figures seem to eliminate such therapy as a factor in the variation of coliform organisms in the urinary tract.

The length of time that the coliform bacilli had been present in the urinary tract was also considered as a possible factor. A study of the histories revealed that the paracolon bacilli were isolated at intervals varying from two days to fourteen years following the first isolation of *Escherichia* or *Aerobacter*. Thus prolonged growth in the urinary tract was eliminated as an important factor in favor of the variation from typical to aberrant coliform organisms.

The cause of this apparent variation and the factors influencing it remain unknown. However, we believe that the evidence which we have presented does suggest that in the infected urinary tract conditions are present which influence and favor the variation of *Escherichia* and *Aerobacter* and result in the appearance of paracolon bacilli in urine cultures.

SUMMARY

1. Paracolon-bacilli appear to be aberrant forms of *Escherichia* and *Aerobacter* which differ from the typical coliform organisms in their failure to ferment lactose promptly with acid and gas. Other variations may also

represent an alteration in the normal fecal flora, possibly as a result of a physiologic disbalance in the individual." Again in regard to the isolation of paracolon bacilli from the infected intestinal tract, Dudgeon and Pulvertaft⁷ state that conditions in the intestines may be such that the rapid multiplication of the slow-lactose-fermenting organisms is allowed. Parr^{11, 10} has demonstrated that in stored feces there is a marked change in the flora resulting in the disappearance of typical *Escherichia* and more especially *Acrobacter* and a marked increase in the slow-lactose-fermenting variants (paracolon bacilli). This change, Parr suggests, is due to the degradation of ordinary, normal coliform organisms.

As far as we have been able to ascertain, the variation of *Escherichia* and *Acrobacter* to paracolon bacilli has never been demonstrated in the urinary tract. We feel, however, that the evidence presented in Table V suggests that such a variation does take place. Since the urinary tract is not the normal habitat of the coliform bacilli, this change in environment may account for the variation. It may also be possible that some factor provided by the infected state of the urinary tract may be responsible for the change from typical to aberrant coliform organisms.

In vitro studies and experiments have furnished considerable conflicting evidence concerning variation in coliform organisms. A number of investigators have been unable to bring about the change from lactose-fermenting to the lactose-negative form. Parr and Robbins¹¹ express the opinion that "the prompt and typical fermenter of that sugar (lactose) is a well-stabilized form in respect to that test." Stewart³³ and Hall³⁴ failed to observe the production of lactose-negative from lactose-fermenting strains. Hershey and Bronfenbrenner³⁵ were unable by serial transfers in synthetic media containing sodium succinate as the sole source of carbon to cause the variation of typical *Esch. coli* to the lactose-negative form, although variants of paracolon bacilli which had reverted to prompt lactose fermenters changed in this medium with the resultant production of lactose-negative strains.

Other investigators have been able to demonstrate the production of aberrant coliform bacilli from typical *Escherichia* and *Acrobacter*. Stuart, Mickle, and Borman³ report the finding of nonlactose fermenting variants in two strains of typical coliform bacilli after two years on agar slants. These variants were antigenically identical with the parent strains. Kriebel⁵ also reports that of 120 strains of *Escherichia* stored for several months on agar slants nineteen gave rise to lactose-negative variants. Lommel²⁸ suppressed lactose fermentation using media containing malachite green and safranin, and Penfold³⁶ obtained lactose-negative strains from coliform bacilli grown on media containing chloracetic acid. This work contributes little information concerning the factors influencing this variation, but these reports do demonstrate that the variation does take place, that paracolon bacilli may arise naturally from coliform organisms (that is, on stored agar slants), or that they may be produced artificially by growth on media containing certain substance.

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occur, so that a large number of culturally different paracolon bacilli may be encountered. The common variations which we have found in paracolon bacilli from urine cultures have been listed, and the cultural types frequently isolated are given in Table II.

2. Culturally paracolon bacilli may resemble *Proteus*, *Salmonella*, *Shigella*, and *Eberthella* and are frequently mistaken for members of these genera. They may usually be differentiated biochemically, but in some instances serologic tests are necessary.

3. In one year, 100 strains of paracolon bacilli were isolated from urine specimens from fifty-seven women patients, representing 3.02 per cent of the total number of urine cultures received in the laboratory during 1945 and 4.3 per cent of the positive cultures.

4. Fifty-one histories were studied to determine the significance of paracolon bacilli in the female urinary tract. In 50.9 per cent of the cases there were symptoms of pyelitis; in 21.5 per cent, symptoms of cystitis. White blood cells only were found in 5.8 per cent of the patients, and 21.5 per cent showed no signs or symptoms of urinary tract infection.

5. In 52.9 per cent of the cases the paracolon bacilli were associated in the urinary tract with either *Escherichia* or *Aerobacter*. If only the instances in which more than one culture was taken are considered, then this association between typical and aberrant coliform bacilli occurred in 87.1 per cent of the cases.

6. It has been suggested that these paracolon bacilli may, in a number of cases at least, be the result of variation in the urinary tract, that in such cases the original infecting agent was a typical *Escherichia* or *Aerobacter*, and that, under conditions as yet undetermined, variation occurred in these typical coliform organisms with the resultant appearance of paracolon bacilli in urine cultures.

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this chemical in suitable combination with bacteriophages seemed to offer advantages, in spite of its toxic effects in older patients. The combination was particularly helpful in the early fulminant stage of staphylococemia. Ordinarily, we found it best to discontinue the sulfathiazole after the first or second week. The newer biologic agent, Fleming's penicillin, offers greater advantages because it can be produced in a potent form which is almost entirely free from toxic action upon the human host even when used by intravenous injection continuously for many months. This agent alone inhibits the growth of many strains of staphylococcus. However, there are strains of this organism which are naturally resistant to penicillin, and some of the susceptible strains are able to acquire a high resistance to this agent after a period of adaptation.

Complete understanding of biologic phenomena is perhaps still beyond our mental capacity, but it now seems well established that the staphylococcus becomes resistant to penicillin in part by elaborating a substance or substances, to which the general name of penicillinases has been given, which neutralize, inhibit, or destroy the bacteriostatic and bactericidal properties of the penicillin. Doubtless all staphylococci produce these substances in some amount, and the strains which have greater resistance to penicillin produce greater amounts of them. Naturally penicillin-resistant strains of bacteria may in part account for the occasional disappointment in penicillin therapy of fulminant staphylococemia, while the acquired resistance would seem to be operative especially in the more chronic infections, such as persistent osteomyelitis.

Before discussing individual clinical examples of conjoined therapy, some of the work with cultures in vitro may be mentioned. Himmelweit² working in the laboratory of Sir Alexander Fleming at St. Mary's Hospital, London, has observed that a strain of staphylococcus moderately resistant to both penicillin and bacteriophage became much more susceptible to destruction when exposed to the action of these two biologic agents in combination. Repetition of Himmelweit's experiments in our laboratory, using our own bacteriophages and cultures obtained from septic patients in our own series, has resulted in essential confirmation of his observations.

Gram-negative bacteria of the coli group are ordinarily resistant to the action of penicillin. However, when combined with bacteriophage, the penicillin exerts a synergistic effect in vitro against cultures of colon bacilli which seems to be analogous to the similar effect on the staphylococcus.

In one instance a strain of colon bacillus (Nbl) obtained in a blood culture was found resistant to penicillin, so that no inhibition of growth was evident in broth containing as much as 5,000 units penicillin per 100 c.c. of medium. When, however, this colon bacillus was grown in broth containing only 50 units penicillin per 100 c.c., the filtrate of this flourishing culture showed peculiar properties. When added to newly inoculated broth, this filtrate caused complete lysis of the Nbl strain at two hours and at four hours followed by secondary clouding at twenty-four hours, and this phenomenon was observed consistently in subsequent serial filtrations, even without further

CONJOINED ACTION OF PENICILLIN AND BACTERIOPHAGES

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IN THE successful healing of an infectious disease, many agencies ordinarily participate. General medication may well be directed to establish those qualities in the internal nutrient media of the body, particularly the blood and lymph, which will favor the successful activity of the human cells and discourage or inhibit the activities of the parasitic organisms, with due consideration of the natural biologic properties and mechanisms for defense and offense inherent in host cell and parasite. The relationships are ordinarily complex rather than simple.

The malignant type of infection with staphylococcus in which the bacterial invader has been able to master the local defenses so as to gain and persist in the circulating blood, septic staphylococcemia, is one example of disease in which there is urgent need of an alteration in quality of the blood and lymph so as to oppose more effectually the infectious agent and perhaps to tip the scale in the conflict in favor of the human host. Even after this has succeeded to the point of eliminating the bacteria from the general circulation, there often remain metastatic localizations of the infection, particularly in bone, kidney, or skin. Suppression of the infection in these focal lesions may require the prolonged maintenance of the special healing qualities in the circulating blood. Many internal remedies have been used, from time to time, in the attempt to improve the blood so as to aid in overcoming the staphylococcus. Various preparations of arsenic, bismuth, mercury, and tin have come down to us from the older therapeutics, and they still have their place. In the more modern era, however, some of the synthetic organic chemicals, particularly sulfathiazole, and some of the biologic agents, bacterial vaccines, bacteriophages, and penicillin, have come into the foreground. The conjoined action of the last two, namely bacteriophage and penicillin, has been remarkably promising in our experience during the last few years.

Our earlier studies of staphylococcemia¹ were concerned especially with the production of potent bacteriophages in such form that they could be administered by intravenous injection in large amounts without serious untoward effects and also with the practical therapeutic use of these bacteriophages in the treatment of septic staphylococcemia, its complications and sequelae, in experimental animals and in human patients. Bacteriophage therapy was employed alone and also in conjunction with neoarsphenamine in the subacute and chronic staphylococcemias. With the advent of sulfathiazole, the use of

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TABLE I

GENERATION	1	2	3	4	5	CONTROL
Meat infusion broth	10.0 c.c.	10.0 c.c.	10.0 c.c.	10.0 c.c.	10.0 c.c.	10.0 c.c.
Fresh coli culture	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Penicillin units	5	5	5	None	None	None
Filtrate 0.5 c.c.	None	Gen. 1	Gen. 2	Gen. 3	Gen. 4	None
Incubation at 37° C.						
Growth cloud						
Two hours	+++	++	0	0	0	+++
Four hours	+++	0	0	0	0	---
Twenty-four hours	+++	++	++	++	++	+++

and usually resistant to bacteriophages. *Streptococcus fecalis* is a notable exception, as we^{3,4} have previously reported. This organism, which is not infrequently the infecting microbe in bacterial endocarditis, is ordinarily very resistant to penicillin and at the same time susceptible to lysis by suitable bacteriophages. More recently we have met with another type of streptococcus, closely resembling *Streptococcus salivarius* but relatively less susceptible to penicillin and capable of lysis by bacteriophage in vitro. Infections with organisms of this type present an opportunity for therapeutic use of penicillin and bacteriophages in combination.

Clinical application of these principles has already been made. Detailed case reports cannot be presented here, but brief summaries in regard to a few patients treated at the Post-Graduate Hospital may serve to illustrate the procedures and the results and perhaps may encourage others to use penicillin and bacteriophages in combination when indicated.

M. R., a 21-year-old white man, was admitted to the hospital for the eighth time on April 8, 1944, with persistent sinus and ulcerations of upper left tibia and chronic osteomyelitis of fourteen years' duration. The detailed record of treatment in the hospital for the period of about two months is too voluminous for presentation at this place, but a brief summary may be of interest. The first intravenous injection at 4:45 P.M. April 8 contained 5 c.c. specific staphylococcus bacteriophage and 5,000 units of calcium penicillin. Subsequently 2 c.c. specific staphylococcus bacteriophage and 5,000 units penicillin calcium were given every two hours by intramuscular injection, 6:30 P.M. April 8 to 6:30 A.M. April 9; then at 8:29 A.M. an intravenous injection of the same dose was given. At 10:25 A.M. April 9, the bacteriophage was increased to 5 c.c. and the penicillin calcium to 10,000 units, given every two hours by intravenous injection during the day from about 8:30 A.M. to 4:30 P.M. and by intramuscular injection during the night. On April 10, because of general malaise, nausea, and headache, the dose was changed at 10:24 A.M. to bacteriophage 2 c.c. and penicillin calcium 20,000 units during the day and bacteriophage 5 c.c. with penicillin calcium 20,000 units during the night, continued to April 18 at 10:21 A.M. At this time the penicillin dose was reduced to 10,000 units every two hours and on April 23 at 8:28 A.M. to 5,000 units. Bacteriophage doses were varied somewhat from 2 to 5 c.c. On May 3 the night doses were reduced to 1 c.c. and the doses during the day continued at 2 c.c. At this time the buttocks were quite sore from the

addition of penicillin. Therefore, a native bacteriophage, latent in the original Nbl strain, had revealed itself as lytic for this strain in the presence of small amounts of penicillin and exhibited lytic effect not only in the culture tube containing penicillin but, after attaining potency in this medium, was able to induce temporary lysis in subsequent serial propagation without penicillin in the medium. Evidently the presence of the penicillin favored the bacteriophage in its action against the host bacterial cell, so that the latent bacteriophage, originally so feeble as to escape recognition, acquired potency against its host cell. These observations are of theoretical interest for the student of bacteriophagy and also of practical significance for the rapid production of a bacteriophage to be used in treatment of a desperately ill patient.

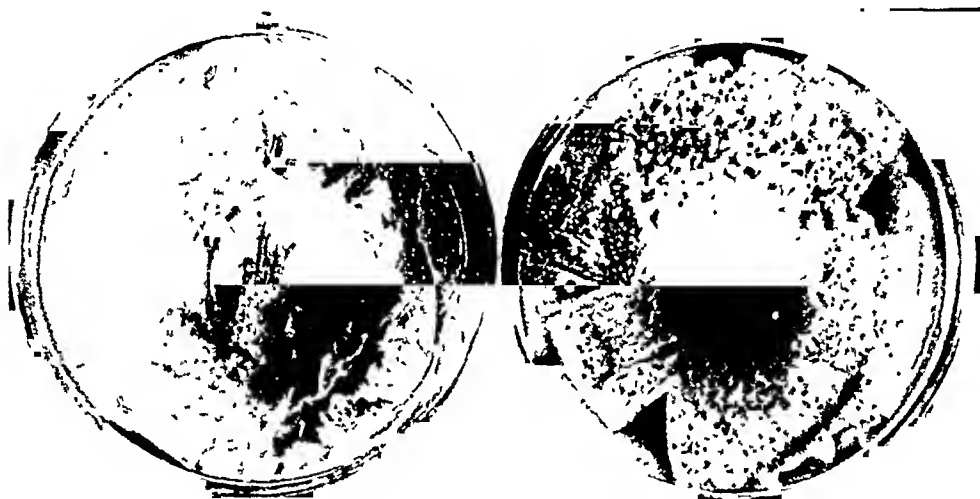


Fig. 1.—Photograph of plate cultures on agar, showing at the left a mass culture of the original Nbl strain and at the right a precisely analogous mass culture of this Nbl strain after the activity of its latent bacteriophage had become enhanced by propagation in the presence of penicillin and serial filtrations.

The protocol of one of these experiments is shown in Table I. The filtrate of the respective preceding generation was used in Generations 2 to 5, with penicillin in Generations 1 to 3 but no penicillin in Generations 4 and 5. In each instance the degree of clouding was estimated by comparison with the control tube recorded as 4 plus at the particular time. In Fig. 1 is shown a photograph of an agar plate on which had been spread 0.05 c.c. of suspension of coli culture, Nbl strain, with subsequent incubation for eighteen hours. Here the growth was confluent without recognizable sign of bacteriophage effect. On the other agar plate there was spread 0.05 c.c. of a precisely analogous suspension of coli culture, Nbl strain, of Generation 5 in the experiment recorded in Table I, with subsequent incubation for eighteen hours. Here the confluent bacterial growth presented the moth-eaten appearance due to the formation of numerous plaques by action of the bacteriophage.

The pathogenic streptococci of the hemolyticus group and of the salivarius group are for the most part susceptible to the bacteriostatic action of penicillin

C. S., a 36-year-old white man was admitted Dec. 3, 1943, with mitral endocarditis due to *Staphylococcus aureus*, the growth of which was inhibited in vitro by 2 units penicillin per 100 c.c. of medium. After treatment with bacteriophage and penicillin to Feb. 28, 1944, the patient was discharged March 18 and has continued to receive bacteriophage injections at intervals. He has remained well and was active at work in May, 1944. This case has been reported.⁵

S. D., a 64-year-old white woman, was admitted to the hospital Dec. 31, 1944, partly disoriented, with temperature of 104.6° F. and harsh systolic murmur over the aortic area, irregular pulse, and extra systolic contractions every three or four beats. Blood cultures taken Dec. 31, 1944, and Jan. 2, 3, and 4, 1945, all developed growth of *Staphylococcus aureus*, which was not appreciably inhibited by 500 units of penicillin per 100 c.c. of medium. It was also moderately resistant to the available bacteriophages. The patient was given penicillin and staphylococcus bacteriophage every two hours from January 2 to February 26, and the bacteriophage was continued twice daily to May 18. She was discharged June 16, 1945, and has remained in good condition. The long record of this patient has been prepared for separate publication.

L. C., a 69-year-old white woman, was admitted to hospital at 4:45 P.M. Aug. 3, 1944, with a large ventral hernia which had become strangulated and gangrenous. There was central crepitation and firm brawny induration about the periphery. She was given 20,000 units of penicillin by intramuscular injection at 5:30 P.M. and then taken to the operating room. The central portion had already ruptured to permit escape of gas. At the operation this spontaneous opening was enlarged, and the perforated loop of colon was drawn out of the wound and supported by a heavy glass rod through the mesocolon and surrounded by several pads to prevent its retraction. The patient was returned to bed in a critical condition. Penicillin, 20,000 units, was given by intramuscular injection at 7:30 P.M. and every two hours during the night. Culture of the exudate from the wound yielded *Clostridium welchii*, *Escherichia coli*, and two types of anaerobic streptococci. At 9:30 A.M. August 4, 0.2 c.c. of coli bacteriophage was given along with the 20,000 units penicillin by intramuscular injection, and this dose was continued every two hours to August 21. At this time she was allowed to dangle her feet. The colostomy continued to function well. On September 23 the portion of transverse colon involved in the colostomy was fully resected, with end-to-end anastomosis of the colon and anatomical repair of the hernia. She was discharged in good condition on Nov. 20, 1944.

M. N., a white woman, born Dec. 8, 1882, had a gastroenterostomy performed in 1934 and a subtotal gastrectomy on June 21, 1945. During a stormy postoperative period of six weeks with temperature range from 99.4 to 105.4° F., she received numerous intravenous infusions of dextrose, sulfadiazine, whole blood, blood plasma, and amigen. On August 3 the patient had an unusually severe chill with rise in temperature to 106.0° F. A specimen of blood taken at 4:00 P.M. on this day yielded growth of approximately 1,900 colonies of *Esch. coli* per cubic centimeter of the patient's blood. Penicillin and coli

many intramuscular injections, and the openings on the leg were practically healed. The patient was allowed to be out of bed. The penicillin was discontinued after 2:25 P.M. May 4, and the bacteriophage continued by intravenous injection 2 c.c. only twice daily to May 17. A small area of ulceration still remained, and so on May 17 the injections every two hours of bacteriophage 2 c.c. and penicillin calcium 10,000 units were started again. The ulcer was also treated locally with undiluted bacteriophage and penicillin 600 units per cubic centimeter for irrigation. The frequent injections were again discontinued on June 11, and only the bacteriophage in dose of 2 c.c. was continued twice daily to June 15. The last crust separated on June 11 leaving a smooth pink epidermis.*

TABLE II

BACTERIOPHAGE (C.C.)		PENICILLIN (UNITS)	BACTERIOPHAGE (C.C.)		PENICILLIN (UNITS)
<i>October 9</i>			<i>October 11 (cont'd)</i>		
2:10 P.M.	5 I.V.	10,000 I.V.	4:15 A.M.		10,000 I.M.
3:10 P.M.	10 I.V.	10,000 I.V.	6:15 A.M.		10,000 I.M.
4:15 P.M.	15 I.V.	10,000 I.V.	8:09 A.M.	10 I.V.	10,000 I.V.
5:18 P.M.	20 I.V.			5 local irrigation	
			10:15 A.M.	10 I.V.	10,000 I.V.
6:15 P.M.		10,000 I.M.	12:14 P.M.	10 I.V.	10,000 I.V.
8:15 P.M.		10,000 I.M.	2:15 P.M.	10 I.V.	10,000 I.V.
10:15 P.M.		10,000 I.M.	4:13 P.M.	10 I.V.	10,000 I.V.
<i>October 10</i>			<i>October 12</i>		
12:15 A.M.		10,000 I.M.	12:15 A.M.		10,000 I.M.
2:15 A.M.		10,000 I.M.	2:15 A.M.		10,000 I.M.
4:15 A.M.		10,000 I.M.	4:15 A.M.		10,000 I.M.
6:15 A.M.		10,000 I.M.	6:15 A.M.		10,000 I.M.
8:10 A.M.	10 I.V.	10,000 I.V.	8:10 A.M.	10 I.V.	10,000 I.V.
10:13 A.M.	10 I.V.	10,000 I.V.		5 local irrigation	
12:15 P.M.	10 I.V.	10,000 I.V.	10:10 A.M.	10 I.V.	10,000 I.V.
2:07 P.M.	10 I.V.	10,000 I.V.	12:10 P.M.	10 I.V.	10,000 I.V.
4:15 P.M.	10 I.V.	10,000 I.V.	2:15 P.M.	10 I.V.	10,000 I.V.
			3:14 P.M.	10 I.V.	10,000 I.V.
6:15 P.M.		10,000 I.M.	<i>October 13</i>		
8:15 P.M.		10,000 I.M.	8:09 A.M.	9 I.V.	
10:18 P.M.		10,000 I.M.		1 local irrigation	
<i>October 11</i>			10:13 A.M.	10 I.V.	
12:15 A.M.		10,000 I.M.	12:19 P.M.	10 I.V.	
2:15 A.M.		10,000 I.M.	2:10 P.M.	10 I.V.	

E. M., a 54-year-old white woman, secretary to a surgeon, was admitted Oct. 9, 1944, with right facial carbuncle of 5 days' duration. On the day of admission it presented brawny edema of the face, threatening extension to the right orbit, and the temperature reached 102.0° F. on this day. After taking blood for sedimentation rate and blood culture and pus from the carbuncle for culture, the patient was treated with bacteriophage and penicillin as shown in Table II. Note that the injections were given intravenously during the day and intramuscularly during the night. The patient was discharged at 4:30 P.M. Oct. 13 in excellent condition and even escaped a visible scar.

*This patient was seen on July 29, 1946. His condition at this time was excellent. There had been no recurrence of ulceration.

concerned require further elucidation. For practical purposes it is significant that this combined action is also manifest in the clinical use of these agents in treating otherwise malignant infections, particularly those due to staphylococci, colon bacilli, and selected members of the streptococcus group.

The brief notes in regard to nine patients serve to indicate the sort of results which have been achieved by the diligent use of these agents in combination. Some of these cases have already been reported elsewhere in more detail. Others of them are of such practical interest that detailed case reports may be expected to appear in the future. Meanwhile, it is hoped that physicians confronted with problems of this sort may be encouraged to undertake the therapeutic programs which have sometimes succeeded in our hands.

SUMMARY

1. Cultures of staphylococcus moderately resistant to penicillin and to bacteriophages may be more effectively inhibited in their growth by a combination of these agents. Similar observations have been made on cultures of colon bacilli and some types of streptococci.

2. Severe types of infection with staphylococcus, such as chronic osteomyelitis, facial carbuncle, and staphylococcal endocarditis have responded to combined therapeutic use of penicillin and bacteriophages.

3. Intestinal perforation associated with general peritonitis and bacteremia has been successfully treated with coli bacteriophage and penicillin.

4. Bacterial endocarditis due to streptococci relatively resistant to penicillin has responded favorably to treatment with penicillin and suitable streptococcus bacteriophage.

We wish to thank Dr. R. L. Preston, Dr. E. C. Brenner, Dr. C. A. Poindexter, Dr. R. McGrath, Dr. R. F. Carter, Dr. T. H. Russell, Dr. H. A. Riley, and Dr. J. W. Hinton, for the opportunity to see and to treat their patients in consultation.

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baeteriophage were then administered to September 2, and the baeteriophage continued to Sept. 19, 1945, when the patient was discharged in good condition. The record of this patient is too long for presentation here.

S. A., a 47-year-old white man, was admitted Aug. 29, 1945, and after careful preoperative preparation the splenic flexure of the colon was resected on September 4 to remove an annular carcinoma. Subsequently the temperature rose and there was a chill on September 6, with signs of pneumonia. Sulfadiazine and penicillin were administered without favorable effect. On September 17 he was disoriented, and there were bubbles of gas in the subcutaneous tissues easily detected by crackling over the chest wall and left upper extremity. In this grave situation stock coli baeteriophage and penicillin were given by injection every two hours. Following abdominal paracentesis on September 20, the peritoneal cavity was irrigated twice daily with penicillin and coli baeteriophage, and after spontaneous rupture of the recently healed surgical wound, the fecal fistula at this site was irrigated twice daily with the penicillin-baeteriophage mixture. There was extensive soiling of the general peritoneal cavity with intestinal contents and free communication between the drain in the paracentesis and the opening of the spontaneous fecal fistula. The penicillin and the baeteriophage were discontinued on November 16, and the patient was discharged to his home on November 19. On May 20, 1946, he appeared to be in excellent condition. The record of this patient is in preparation for presentation elsewhere.

P. M., a 34-year-old white man, was admitted Jan. 23, 1944, and discharged Nov. 16, 1944. He had bacterial endocarditis with both *Str. fecalis* and *Str. salivarius* found in cultures of his blood. He was treated with enterococcus baeteriophage, penicillin, and sodium bismuth thioglycollate. The case report has already appeared.⁶ We may add that the patient remained in good condition, actively engaged in business in June, 1946.

J. P., a 27-year-old white man, was admitted July 21, 1945, by transfer from another hospital where he had been treated for bacterial endocarditis with sulfadiazine and penicillin over a period of four months, during which time the bacterial infection of the blood stream continued. The streptococcus of his blood culture required 8 units penicillin per 100 c.c. to inhibit macroscopic growth. The patient was treated with penicillin, baeteriophages, and sodium bismuth thioglycollate and was finally discharged in good condition on Feb. 11, 1946. He was actively engaged in business in June, 1946. The long record of this patient is in preparation for separate presentation.

COMMENT

The recent additions to the therapeutic agents available for internal medication in infectious diseases have improved in a marvelous fashion the outlook for success in dealing with these conditions. The biologic agents, penicillin, and the baeteriophages are not antagonistic to each other, and in many instances they can be shown to exert a synergistic effect upon the bacterial culture in the test tube. This combined action in the test tube may be designated as a potentiating, conjoined, or synergistic effect. The precise mechanisms

TABLE I. MAIN AND GROUP AGGLUTININS IN DIFFERENT VARIETIES OF TYPHUS⁸

	OX-19	OX-2	OX-K
Epidemic typhus	+++	+	--
Endemic typhus	+++	+	--
Scrub typhus	--	--	+++
Rocky Mountain spotted fever	+	+	+

in either epidemic or murine typhus and that the OX-2 and OX-K agglutination is equal in titer to that found for the OX-19. Since these results are at variance with those observed in this laboratory, it was considered worth while to present out data. While specific serologic reactions are now available for the diagnosis of Rocky Mountain spotted fever,⁹ the Weil-Felix agglutination test still remains an important laboratory aid. Its value, however, depends upon the proper interpretation of the results obtained.

The data presented in Table II represents the results of Weil-Felix tests obtained from a series of cases of Rocky Mountain spotted fever occurring in the United States Army personnel. The laboratory diagnosis was established in each case either by the isolation of the infectious agent from the blood of the patient or by the demonstration of the presence of specific complement-fixing antibodies of Rocky Mountain spotted fever.⁹

The Weil-Felix tests, using controlled nonmotile proteus strains of OX-19, OX-2, and OX-K, were performed according to the technique described in *Diagnostic Procedures and Reagents*.¹⁰ The complement fixation tests were performed according to the method previously described.⁹

An analysis of these data would indicate that the most frequent proteus agglutination pattern in cases of Rocky Mountain spotted fever is a high OX-19 and a lower or negative OX-2 titer (eleven cases). This is followed in frequency by a high OX-2 titer and a lower or negative OX-19 agglutination (four cases), a low or negative OX-19 and a negative OX-2 (three cases), and finally, one case in which equal titers were observed. These results are comparable to those reported by Spencer and Maxcy⁴ and Davis and Parker.⁵⁻⁷

It should be observed that 21 per cent of the patients in this series showed a high OX-2 titer and a lower or negative OX-19 agglutination. This combination has not been reported as occurring in either epidemic or murine typhus nor have we found it in a series of such patients from whom serial specimens were examined.^{11, 12}

While the response to the proteus antigens may vary in individual cases, a common pattern or combination of results can be described as being characteristic for each rickettsial disease. The usual reactions observed in epidemic and murine typhus are characterized by a rise in OX-19 titer, which in certain instances may reach high levels. In these cases the OX-2 titer, when it occurs, is lower than the OX-19, and the OX-K is negative. This conforms to the results as recorded by Felix.⁸ In Rocky Mountain spotted fever the OX-19 titer may be high and the OX-2 lower or negative. In these cases the OX-19 titer may reach levels comparable to those ordinarily found in either epidemic or murine typhus. When the OX-19 titer is lower or negative and the OX-2 higher, we are justified in suspecting a case of Rocky Mountain spotted fever. Indeed,

THE INTERPRETATION OF THE WEIL-FELIX AGGLUTINATION TEST IN ROCKY MOUNTAIN SPOTTED FEVER

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AFTER the significance of the Weil-Felix agglutination in epidemic typhus was demonstrated,¹ an attempt was made to ascertain whether this reaction occurred in another rickettsial disease, namely, Rocky Mountain spotted fever. While Kelly,² in 1923, obtained negative results with a proteus OX-19 strain in serum specimens from eight patients with Rocky Mountain spotted fever, Kerlee and Spencer,³ in 1923, reported that the serum from some patients developed agglutinins with OX-19 strains which were far in excess of those encountered in control specimens. In four cases of Rocky Mountain spotted fever, where single specimens were obtained during the febrile period or in early convalescence, titers of from 1/320 to 1/2560 were observed. In one case, where serial specimens were available, a definite rise in titer with an OX-19 strain was recorded. In 1930, Spencer and Maxey,⁴ employing both OX-19 and OX-2 strains in a series of four patients from whom serial specimens were examined, observed one patient who showed a high OX-19 (1/1280) and a low OX-2 titer (1/160) early in the disease, followed by a low OX-19 (1/80) and a higher OX-2 titer (1/640) in early convalescence. The second patient showed a high OX-19 titer (1/2560) and no agglutinins with an OX-2 strain; the third patient developed low titers for both OX-19 (1/40) and OX-2 (1/160); and the fourth patient showed a high OX-19 (1/2560) and a high OX-2 (1/640) titer. Davis and Parker,⁵ in 1932, studied a series of patients from whom single specimens were examined and observed a high OX-19 and a low OX-2 relationship or a low OX-19 and a high OX-2 titer. This was also observed in patients from whom serial specimens were examined. These authors stressed the fact that relatively high titers with an OX-2 strain were occasionally encountered. In 1934, Davis, Parker, and Walker⁶ again reported a series of patients in whom various OX-19: OX-2 relationships were observed, and the same authors,⁷ in 1938, reported two patients from whom rickettsial strains were isolated who showed high OX-2 and low OX-19 titers. It had thus been shown that an OX-19 agglutination could be obtained in cases of Rocky Mountain spotted fever and that occasionally higher titers with an OX-2 strain were present.

In 1933, Felix⁸ prepared a table (Table I) representing the main and group agglutinins in different varieties of typhus. Data obtained from this table have been extensively quoted. According to these findings, it would appear that the OX-19 titer in Rocky Mountain spotted fever is lower than that found

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the presence of an OX-2 agglutination titer of 1/320 or higher would lead us to suspect a case of Rocky Mountain spotted fever. However, since the Weil-Felix proteus agglutination is considered a nonspecific test, the final laboratory diagnosis will depend upon the isolation of the infectious agent or the demonstration of specific antibodies.¹³ In scrub typhus the OX-19 and OX-2 agglutination are negative, and a rise in OX-K titer is usually, but not always, present.^{14, 15} A rise in OX-K titer was formerly regarded as being characteristic of scrub typhus alone, but Zarafonetis, Ingraham, and Berry¹⁶ have recently shown that this can occur in louse-borne relapsing fever. The proteus agglutination reactions in "Q" fever are all negative.^{17, 18}

TABLE III. USUAL WEIL-FELIX AGGLUTINATION REACTIONS OBSERVED IN RICKETTSIAL DISEASES

	ox-19	ox-2	ox-k
Epidemic typhus	++++	+	0
Murine typhus	++++	+	0
Scrub typhus	0	0	+++
"Q" fever	0	0	0
Rocky Mountain spotted fever	++++	+	0
	+	++++	0

The results, as recorded in Table III, are suggested as representing the usual Weil-Felix agglutination results occurring in cases of epidemic and murine typhus, scrub typhus, "Q" fever, and Rocky Mountain spotted fever.

SUMMARY

The usual Weil-Felix agglutination found in Rocky Mountain spotted fever is characterized by a high OX-19 and a lower or negative agglutination with an OX-2 strain and a negative OX-K. In these cases the OX-19 titer may reach levels equal to those found in either epidemic or murine typhus. The next most frequent combination is a high OX-2 titer and a lower or negative OX-19 agglutination. Since this grouping has not been described as occurring in cases of epidemic or murine typhus, we are justified in suspecting a case of Rocky Mountain spotted fever when it is present. The final laboratory diagnosis, however, will depend upon the isolation of the infectious agent or the demonstration of a rise in titer of specific antibodies. A table is suggested as representing the usual Weil-Felix agglutination reactions observed in rickettsial diseases.

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TABLE II. WEIL-FELIX AGGLUTINATION IN CASES OF ROCKY MOUNTAIN SPOTTED FEVER

CASE	DAY OF ILLNESS	WEIL-FELIX		COMPLEMENT FIXATION ROCKY MOUNTAIN SPOTTED FEVER
		OX-19	OX-2	
1	21	0	1/1280	0
	31	0	1/320	0
	40	1/80	1/80	+
2	7	0	0	+
	23	0	0	+
3	10	1/160	1/2560	+
	14	1/160	1/1280	+
4	4	1/40	0	0
	5	1/80	0	0
	13	1/2560	1/80	+
5	17	1/2560	1/640	0
	35	1/320	1/160	+
6	7	0	0	0
	10	0	1/160	0
	24	0	1/640	+
	51	1/80	1/80	+
7	17	1/640	0	+
	34	1/1280	0	+
	65	1/640	0	+
8	9	0	0	0
	16	0	0	+
	18	1/160	1/80	A/C
9	5	0	0	0
	18	1/320	1/320	+
	45	1/40	1/80	+
10	9	0	1/40	0
	12	1/160	1/1280	+
11	4	0	0	+
	9	1/640	1/160	+
12	12	0	0	0
	16	1/2560	1/160	+
	21	1/1280	1/160	+
	31	1/1280	0	+
13	6	0	0	0
	9	0	0	+
	18	1/40	0	+
14	5	0	0	0
	12	1/640	0	+
15	7	1/160	0	0
	14	1/640	0	0
	15	1/640	0	-
	21	1/640	0	+
16	13	1/40	0	0
	25	0	0	+
17	8	0	0	-
	13	0	0	-
	23	1/2560	0	+
	37	0	0	+
18	9	1/320	1/40	+
	28	1/2560	1/80	+
19	7	0	0	0
	13	1/640	1/320	0
	20	1/1280	1/640	0
	140	-	-	+

SULFONAMIDES AND DARK ADAPTATION

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FOLLOWING the description by Bucy¹ of a case of optic neuritis resulting from the administration of sulfanilamide, various visual disturbances have been described following sulfonamide therapy.

Since 1939, a number of transient myopias have been reported in the literature.²⁻¹³ The majority of these cases were due to sulfapyridine, while sulfathiazole and sulfadiazine seemed innocuous in this respect. The pathogenesis of these disturbances is obscure, but in 62 per cent of the reported cases the myopia was brought on by usual therapeutic doses (from 2 to 10 Gm.) of the responsible agent. Mydriasis usually accompanied the myopia, and treatment with atropine and homatropine could not be shown definitely to alter its course. However, when administration of the drug was stopped, vision gradually returned to normal.

Cataracts, retinitis, reduction of the visual fields, blurred vision, and changes in accommodation have variously been reported in isolated cases of patients receiving sulfonamides. Alvaro,¹⁴ Rosenthal,¹⁵ and Reynolds, Evans, and Walsh¹⁶ have noted the effects of sulfonamides on visual fields, visual acuity, light and color sense, accommodation, muscle balance. For the most part, all these authors found some reduction in the width and length of visual fields, as well as a decreased power of accommodation. None of these authors, however, reported any marked change in visual acuity, and, so far as we have been able to ascertain, the literature does not contain a reference to the effects of sulfonamides on dark adaptation.

It is common knowledge that some persons who require therapy with sulfonamides (pilots, bombardiers, motorists, locomotive engineers) have been told not to follow their profession during a course of these drugs. It has been taken for granted, it is presumed, that the reason for such a statement has had to do with some decrease in the visual efficiency of such individuals when they have been so treated. For this reason we are reporting our experiments on the effects of three commonly used sulfonamides upon dark adaptation.

For the determination of the dark adaptation time, a Feldman adaptometer¹⁷ was used. For each test the following technique was routinely adopted: The subjects were requested to avoid bright lights before the test. For ten minutes they sat quietly in a semidarkened room. The test was performed in a dark room. For three minutes the subjects looked at a bright light. This was then turned off, and the time required to identify the direction of an

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noted that all values fall well within the range of five minutes characteristic of normal night vision when tested with this apparatus (Feldman²⁰). They also fall within the more narrow range of normal values given by Hecht and Mandelbaum²¹ for test lights of the same brightness.

As the subjects used in these experiments were in normal health throughout the experiment and were mostly trained to observe and note their own feelings, it is interesting to report the symptoms evidenced by them during their medication period.

In Group B, with the exclusion of two slight headaches, no untoward symptoms were reported. All subjects of Group A receiving sulfathiazole witnessed paresthesia of the tongue; this took the form of tingling and numbness and sometimes disturbances of taste. Subjects of Group C were most affected of all. Without exception they complained of headaches, nausea, dizziness, and general malaise throughout the entire medication period. One subject vomited and discontinued the drug after the first day. In three others, although there was no vomiting, the symptoms were so pronounced that they led to the discontinuing of the drug after the second day. Four cases reported paresthesias of feet and legs. In all three groups mental slowness and some disturbance of memory was a frequently reported symptom.

SUMMARY

1. Results on eighteen healthy individuals indicate that neither sulfathiazole, sulfadiazine, nor sulfanilamide significantly alters dark adaptation time as measured by the Feldman adaptometer.

2. There is an indication, however, that those subjects which received sulfanilamide exhibited enough increase in their dark adaptation times to suggest a possibility that this sulfonamide may have a direct effect upon dark adaptation.

3. These experiments would not corroborate the general opinion that persons taking small doses of sulfonamides daily need necessarily be concerned about visual acuity or efficiency.

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oblong test light of uniform blue-violet color was determined as the dark adaptation time. The brightness of the lights was about 2.3 lamberts for the exposure light and log 6.18 $\mu\mu$ lamberts for the test light.* For the identification of the test light, the subject sat at reading distance from it and was requested to direct both eyes toward a "fixation point" in order that the threshold be taken on a predominantly rod area.

Using this method, the dark adaptation time of eighteen normal volunteers of both sexes was determined before, during, and after medication with sulfathiazole, sulfadiazine, and sulfanilamide.

The subjects were divided into three groups of six. Group A received 4 Gm. of sulfathiazole daily for three days and Group B a similar dosage of sulfadiazine. In Group C, two subjects received 4 Gm. of sulfanilamide, but in four cases the medication period was shortened because of toxic manifestations such as nausea, vomiting, and general discomfort.

The drugs were taken in four doses at the following times: 8:30 and 11:30 A.M., 2:00 and 4:00 P.M. *Adaptometer tests were made three times daily at fifteen minute intervals between 5:00 and 7:00 P.M. before and after (control period), as well as during, the medication period.* Blood samples were taken during the medication period on the first and third days immediately after the completion of adaptation time determinations. Free and total blood sulfonamide concentration was determined by the method of Bratton and Marshall.¹⁹

TABLE I

GROUP	DRUG	DARK ADAPTATION TIME IN SECONDS		BLOOD SULFONAMIDES (FREE MG. %)
		CONTROL PERIOD	MEDICATION PERIOD	
A	Sulfathiazole	49 \pm 20	57 \pm 22	6.0 \pm 1.3
B	Sulfadiazine	42 \pm 22	37 \pm 19	7.6 \pm 1.4
C	Sulfanilamide	44 \pm 23	67 \pm 46	7.0 \pm 0.8

The results are summarized in Table I. As shown by the standard deviations, the values for dark adaptation times in each group were somewhat variable. This was true not only for the data treated as a whole in each group but also for the determinations made on each single subject. This is not surprising considering the multiple influences, psychologic and other, that play upon the subject endeavoring to spot the test light and estimate its direction in minimum time. With practice, however, results became more uniform, and the time required for dark adaptation decreased. This trend was apparent in all three groups.

Under the conditions of our experiments, no group revealed a statistically significant difference between the adaptation times of the control and medication periods. However, in two subjects of Group C, the dark adaptation times were consistently elevated during the medication period. It appears difficult to evaluate at present the significance of these changes. It should be

*This was erroneously printed as log 1.6 μ lambert in the abstract (Federation Proc. 4: 135, 1944).

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LIVER FUNCTION TESTS IN NEUROSYPHILITIC PATIENTS WITH INDUCED VIVAX MALARIA OF PACIFIC AND MEDITERRANEAN ORIGIN

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MEDICAL CORPS, CAPTAIN LESTER D. ELLERBROOK AND MAJOR WILLIAM
B. HESSELBROCK, SANITARY CORPS, CAPTAIN WILLIAM W. ENGSTROM
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IN AN earlier paper¹ it was reported that only mild, transient disturbances of liver function were noted in patients with naturally acquired, relapsing vivax malaria of Pacific origin. All of these soldiers had been given quinaerine promptly to terminate their acute attacks. In this paper are presented the findings in 138 soldiers with neurosyphilis whose induced malarial fever was terminated with quinaerine only after they had had on the average eight to fourteen paroxysms and about twenty days of parasitemia.²

SUBJECTS AND METHODS

The subjects were 138 soldiers with neurosyphilis who had completed at least one standard course of antisyphilitic chemotherapy. Prior to inoculation with vivax malaria of Pacific or Mediterranean origin by the bites of laboratory-bred, infected, domestic, anopheline mosquitoes or by the intravenous injection of five to 7 c.c. of blood containing trophozoites, tests of liver function were made. The tests were repeated during the course of the malaria and at varying times following termination of the malaria with quinaerine (2.8 Gm. in 6 days). The patients had an average of forty hours of fever over 104° Fahrenheit.

The following tests were performed: bromsulfalein, cephalin flocculation, galactose tolerance, intravenous hippuric acid, icterus index, serum bilirubin, and urine urobilinogen. The details of the methods used have been given in an earlier paper.¹

RESULTS

Bromsulfalein Test.—The bromsulfalein test was carried out in 138 neurosyphilitic patients (Table I). The amount of dyestuff injected was 5 mg. per kilogram of body weight, and retention of up to 4 per cent at forty-five minutes was considered normal.¹ Prior to fever therapy 138 patients were tested, and 117, or 85 per cent, gave normal findings. Twenty-one men had abnormal values including ten with 6 per cent retention, eight with 8 per cent, one with 10 per cent, one with 12 per cent, and one with 40 per cent. Following fever therapy (from 10 to 100 days later) sixty-six of these 138 men were re-examined, and in fifty-eight men, or 88 per cent, the results were normal. The eight men with abnormal values included two with 8 per cent retention, one

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Galactose Tolerance Test.—The galactose tolerance test was performed on 106 patients prior to malarial therapy, and only one of these excreted over the accepted highest normal value of 3 Gm. of galactose in five hours. No tests were done during fever therapy. Following fever therapy thirty-one men were retested, and all findings were normal. It so happened that the patient with a slightly abnormal value before fever therapy was not re-examined after therapy.

Intravenous Hippuric Acid Test.—The intravenous hippuric acid test was performed on twenty-five patients. Results were reported in terms of hippuric acid in the urine specimen voided one hour after the intravenous injection of 1.77 Gm. of sodium benzoate. A value below 0.80 Gm. was considered abnormal.¹ Prior to fever therapy all values were normal. After a minimum of fifteen hours of temperature of not less than 104° F., three of twenty-two men who were retested had abnormal findings. From thirty to eighty days after disappearance of fever, twelve of the twenty-five men were retested, and ten were normal. The other two men had abnormal values during fever, and on this occasion following fever one still remained abnormal. In two of the three men with abnormal results during the period of fever therapy, there was also definite evidence of brom-sulfalein retention, namely 36 and 40 per cent, respectively. Following fever therapy the bromsulfalein values were normal in these two patients.

TABLE III. ICTERUS INDEX, SERUM BILIRUBIN, AND URINE UROBILINOGEN IN PATIENTS WITH NEUROSYPHILIS BEFORE, DURING, AND IMMEDIATELY AFTER MALARIAL FEVER THERAPY

	SEVEN DAYS BEFORE FIRST POSITIVE SMEAR	PERIOD OF PARASITEMIA		PERIOD OF SIX DAYS' QUINACRINE TREATMENT									
		SEVEN DAYS BEGINNING WITH FIRST POSITIVE SMEAR	SEVEN DAYS BEFORE QUINA- CRINE TREAT- MENT	FIRST THREE DAYS	LAST THREE DAYS								
<i>Icterus Index</i>													
Number of pa- tients	12	32	32	32	32								
Number of tests	12	44	57	53	34								
Per cent tests > 8	8	12	38	47	15								
<i>Serum Bilirubin</i>													
Number of pa- tients	13	32	32	32	32								
Number of tests	13	45	63	54	33								
Per cent tests > 0.50	7	7	17	24	12								
<i>Urine Urobilinogen</i>													
	SEVEN DAYS BEFORE FIRST POSITIVE SMEAR	SEVEN DAYS BEGINNING WITH FIRST POSITIVE SMEAR	SEVEN DAYS BEFORE QUINACRINE TREATMENT	SIX DAYS OF QUINACRINE TREATMENT									
		1 2 3 4 5 6 7	7 6 5 4 3 2 1	1 2 3 4 5 6	6 5 4 3 2 1	1 2 3 4 5 6	6 5 4 3 2 1	1 2 3 4 5 6	6 5 4 3 2 1	1 2 3 4 5 6	6 5 4 3 2 1		
Number of pa- tients	32	18 21 21 25 21 21 22	20 18 22 24 26 25 25	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23		
Number of tests	92	18 21 21 25 21 21 22	20 18 22 24 26 25 25	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23	25 24 28 23 27 23		
Per cent tests > 1:20	0	6 5 14 4 14 19 41	25 28 36 38 35 40 40	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6	36 46 25 9 15 6		

TABLE I. BROMSULFALEIN TESTS IN PATIENTS WITH NEUROSYPHILIS BEFORE, DURING, AND AFTER MALARIAL FEVER THERAPY

RELATION TO FEVER THERAPY	BEFORE	DURING (15 TO 40 HOURS OF FEVER)	AFTER (10 TO 100 DAYS LATER)
Number of patients	138	18	66
Number of tests	138	18	66
Patients with retention of 6 per cent or greater			
Number	21	15	8
Per cent	15	83	12

with 10 per cent, two with 12 per cent, one with 16 per cent, one with 32 per cent, and one with 40 per cent. Of the 138 patients only eighteen were tested before, during, and after fever therapy. Fourteen of these men had normal values in the pre-fever study, while three had 6 per cent retention and one 8 per cent. During fever therapy fifteen of these men had abnormal values with those in ten ranging between 12 and 40 per cent. Following therapy fifteen of the eighteen patients had normal values, while of the remaining three with abnormal values one had 10 per cent retention, one had 12 per cent, and one had 32 per cent.

Cephalin Flocculation Test.—A series of 256 sera from forty patients (Table II) were tested with the Wilson antigen, and 181 sera from thirty-six of these same patients were tested with the Difco antigen. Before inoculation with malarial parasites and from inoculation to the finding of the first positive smear for malaria, no 3 or 4 plus results were obtained. During the next four days 12 per cent of the results with the Wilson antigen and 15 per cent with the Difco antigen were 3 or 4 plus. After the fifth day there was a sharp increase

TABLE II. CEPHALIN FLOCCULATION TESTS IN PATIENTS WITH NEUROSYPHILIS BEFORE AND DURING MALARIAL FEVER THERAPY

TIME OF TESTS WITH RELATION TO MALARIA	PERCENTAGE OF 3 AND 4 PLUS TESTS	
	WILSON ANTIGEN (256 TESTS IN FORTY PATIENTS)	DIFCO ANTIGEN (181 TESTS IN THIRTY-SIX PATIENTS)
Before inoculation	0	0
Inoculation to first positive smears	0	0
During days of positive smears		
1 to 4	12	15
5 to 7	35	33
8 to 11	70	94
12 to 14	89	96
15 to 18	97	100
19 to 21	100	100

in positive results, so that within a period of from fifteen to eighteen days with the Difco antigen and from nineteen to twenty-one days with the Wilson antigen all had a 3 or 4 plus test which persisted during the next week in those individuals who were tested. No tests were performed following fever therapy. It is noteworthy that in the present studies the sensitivity of the Wilson and Difco antigens was about the same, whereas in the previous study¹ the Difco antigen at that time was more sensitive.

WITH SPOOROZOITE INDUCED PACIFIC MALARIA

DAYS OF QUINACRINE TREATMENT												17 TO 70 DAYS AFTER QUINACRINE TREATMENT		
FINDINGS FOURTH WEEK			FIRST			SECOND			FOURTH			BROM- SULFALEIN	HIPPOURIC ACID	GALAC- TOSE
G. I. SYMPTOMS			ICTERUS INDEX	BILIRUBIN	UROBILIN- INOGEN	ICTERIC INDEX	BILIRUBIN	UROBILIN- INOGEN	ICTERUS INDEX	BILIRUBIN	UROBILIN- INOGEN			
NAUSEA	VOMIT- ING													
Yes	Yes		7	0.35	1:5	7	0.30		4	0.10	1:10	8%		
Yes	No											2%		1.12
Yes	Yes											Trace	0.99	
Yes	Yes		8	0.25	1:5	6	0.20	1:5			1:5	Neg.		
Yes	No		10	0.35	1:5	8	0.35	1:10	7	0.10	1:5	Trace		1.37
Yes	Yes					15	0.75		9	0.35	1:20	2%		0.86
No	No		7	0.25	1:200	8	0.40	1:50	7	0.25	1:5	4%		1.05
Yes	Yes					30		1:100	15	2.00	1:200	4%		0.65
Yes	Yes											Trace	1.06	
Yes	No		8	0.35	1:5	6	0.30	1:5	5	0.10	1:5			
No	No		8	0.40	1:5	6	0.30	1:5	6	0.10	1:5	Trace		0.69
Yes	Yes		12	0.80	1:5	10	0.50		7	0.20	1:5	4%		0.43
Yes	Yes		12	0.60	1:20	8	0.30	1:50	7	0.35	1:5	10%		0.16
Yes	Yes		10	0.45	1:5	11	0.60	1:50	6	0.10	1:5	Trace		1.33
Yes	Yes		10	0.40	1:20	10	0.65	1:200	7	0.40	1:20	Neg.		0.66
Yes	No		50	7.00	1:50	50	4.50		30	1.40	1:100	2%		0.20
Yes	Yes				1:250	65	4.90				1:100	2%		1.01
Yes	Yes		40	3.50	1:500	16	0.65	1:100			1:20	2%		
No	No											2%		
Yes	No				1:5	10	0.55	1:5			1:5	Trace		2.34
Yes	Yes		6	0.30		8	0.45	1:5	6	0.20	1:5	2%		0.76
Yes	No				1:100			1:200	16	1.00	1:100	4%		0.57
Yes	No		10	0.35	1:20	10	0.45	1:20	7	0.20	1:5	Trace		0.76
No	No												0.97	
Yes	Yes		9	0.55	1:50	9	0.80	1:20	7	0.20		Neg.	0.96	
Yes	No		12	0.85	1:5	12	0.90	1:100	7	0.20	1:10	4%		0.18
Yes	Yes		8	0.35	1:50	12	0.55	1:500	6	0.10		2%		
No	No											4%		
Yes	Yes		7	0.30	1:5	6	0.10	1:5	6	0.10	1:5	2%		
Yes	Yes											2%		
Yes	Yes												0.58	
Yes	Yes											40%	0.59	
32	32	18	18	20	22	21	18	19	19	21	29	6	17	
84	59	56	33	35	60	48	50	21	16	19	10	33	0	

c.c. of blood; bromsulfalein, in per cent retention at forty-five minutes after the giving of 5 mg. of injection of 1.77 Gm. of sodium benzoate; galactose, in grams excreted in the urine in five hours after

tion of fever by quinaerine. The increase carried over into the first three days of quinacrine treatment during which abnormal values rose still further to 47 and 24 per cent, respectively. However, during the next three days abnormal values dropped to 15 and 12 per cent, respectively.

Urine Urobilinogen.—The test for urine urobilinogen was performed on fresh morning specimens. A positive test in a dilution greater than 1:20 was considered to be abnormal and is herein referred to as a positive test. Examinations of the urine were carried out in the same thirty-two men on whom icterus

TABLE IV. LIVER FUNCTION TESTS IN THIRTY-TWO NEUROSYPHILITIC

PATIENTS	INITIAL STUDIES					DURING PERIOD OF PAROXYSMS						
	ICTERUS INDEX	BILIRUBIN	BROM-SULFAL- EIN	HIP- PURIC ACID	GALACTOSE	FIRST WEEK			SECOND WEEK			SECOND TO FOURTH WEEK
						ICTERUS INDEX	BIL- RUBIN	UROBIL- INOGEN	ICTERUS INDEX	BIL- RUBIN	UROBIL- INOGEN	
1	6	0.10	6%		Neg.	6	0.10	1:5	6	0.15	1:20	
2	15	0.70	2%		0.56		0.50	1:10	7	0.25	1:50	
3	5	0.25		0.97	0.87							14%
4	5	0.10	4%		1.25	7	0.30	1:5	5	0.20	1:5	0.61
5	8	0.40	Neg.		1.32	6	0.30	1:5	8	0.35	1:20	
6	8	0.35	Trace		1.11		0.30	1:5	10	0.35	1:50	
7	9	0.40	2%		0.32		0.15	1:5	9	0.35	1:5	
8	8	0.40	4%		0.94	6	0.20	1:5	12	0.90	1:50	
9	12	0.40	2%	1.05	0.18							
10	6	0.20	2%	0.90		6	0.20	1:100			1:10	
11	8	0.30	2%		0.58			1:10	8	0.30	1:5	
12	6	0.30	2%		1.02	6	0.25	1:10	20	2.00	1:100	
13	7	0.30	6%		0.63	6	0.25	1:10	12	0.80	1:100	32, 40%
14	7	0.30	2%		2.13	7	0.20	1:20	9	0.70	1:500	12, 8%
15	6	0.30	Trace		1.63	7	0.20	1:5	7	0.30	1:50	
16*	5	0.10	Trace		0.45	14	0.50	1:50	50	8.00	1:100	
17*	7	0.20	2%		0.71			1:50	18	1.00	1:100	
18*	7	0.30	2%		0.56	6	0.10	1:5	6	0.25	1:50	40%
19	7	0.40	6%	0.92								32, 8%
20	6	0.20	Trace		1.74	7	0.20	1:10	9	0.35	1:200	0.65
21	7	0.30	2%		0.49	6	0.20	1:20	12	0.75	1:10	
22	8	0.40	Trace		0.82		0.20	1:10		0.10	1:50	
23	6	0.40	Neg.		1.04			1:20	9	0.65	1:50	
24	6	0.20	Trace	0.77								
25	7	0.45	Trace	0.67	1.09			1:5	10	0.65	1:50	4%
26	7	0.20	2%		0.64	14	1.00	1:20	12	0.65	1:50	0.67
27	9	0.20	Trace		0.63	10	0.30	1:500	10	0.65	1:500	
28	9	0.40	2%	1.05								6, 6%
29	6	0.20	2%		0.62	6	0.20	1:20		0.20	1:200	0.95
30	6	0.40	2%	0.83								32, 8%
31	9	0.70		0.89	0.79	7	0.45	1:5				0.65
32	20	2.50		0.87	1.88							0%
32†	32	32	29	10	27	17	21	25	21	23	24	14
‡	22	9	10	20	0	18	5	16	67	48	71	86
												71

The results shown in this table are expressed as follows: icterus index, in units; bilirubin, in milligrams the dye per kilogram body weight; hippuric acid, in grams excreted in the urine in one hour after the intra- giving of 40 Gm. of galactose by mouth; urobilinogen, highest dilution of urine giving a positive test.

*Developed clinical jaundice.

†Number of tests.

‡Per cent abnormal.

Icterus Index and Serum Bilirubin Concentration.—Sera were taken from thirty-two patients for determination of the icterus index and bilirubin concentration of the same specimens (Table III). A value of over 8 units was considered as abnormal for the icterus index and over 0.5 mg. per 100 c.c. for the serum bilirubin concentration.¹ In the seven-day periods before and following the first positive smear, a maximum of 12 and 7 per cent of abnormal values was reached for the icterus index and serum bilirubin, respectively, with a rise to 38 and 17 per cent of abnormal values in the last seven days before termina-

previously been shown in this laboratory that South Pacific vivax malaria, which was to be used therapeutically in many of these patients, disclosed some evidence of transient disturbance in liver function during recurrent attacks but gave little or no indication of permanent hepatic dysfunction; and (4) the patients could be observed both clinically and by laboratory tests over long periods of time, so that the data gathered could be evaluated and compared with current reports of therapeutic malaria of American origin.

It has been stated by Fredricks and Hoffbauer³ that the postmalarial death of a 46-year-old woman under their observation sharply focused their attention on the scant evaluation in the literature of hepatic function in therapeutic malaria. These authors investigated thirty-one patients with neurosyphilis inoculated intravenously with benign tertian malaria. No manifest liver disease was noted prior to malarial therapy. Hepatic function was evaluated by several types of tests. These authors felt that all of their patients adequately studied showed some evidence of hepatic dysfunction but that the abnormalities reported were not presumed to indicate irreversible hepatic damage and the occurrence of such changes did not constitute a reason for avoiding the use of a well-tried and effective therapeutic agent. Kopp and Solomon⁴ studied the liver function in fifty-one patients with neurosyphilis (previously given tryparsamide over long periods of time) treated with malarial fever, artificial fever, or fever induced by typhoid vaccine. Their impression was that following such therapy some alteration of liver function may develop but in most instances is only of mild degree.

In our series of patients with neurosyphilis, Pacific and Mediterranean vivax malaria was induced both by sporozoite and blood inoculation. In general, the results of our serial studies of liver function using malaria of foreign origin are similar to those cited^{3, 4} in the use of malaria of American origin. Before fever therapy a small percentage of our patients showed slightly abnormal values with the bromsulfalein and serum bilirubin tests but with no other tests. The abnormalities were possibly related either to previous antisyphilitic chemotherapy or to syphilis itself. During fever therapy bromsulfalein retention occurred in most patients studied with values ranging up to 40 per cent. Abnormal values were also observed during fever therapy with the cephalin flocculation test (all 3 or 4 plus) and with the icterus index, serum bilirubin, and urobilinogen tests. After termination of fever therapy with quinaerine administration, the tendency was for the results in practically all types of tests to be normal within approximately two months.

SUMMARY

The findings obtained in the present study may be summarized as follows:

1. The results of the bromsulfalein test showed that:

- (a) In 138 patients tested prior to fever therapy, fifteen per cent had abnormal values with only three individuals having 10 per cent or more retention.

- (b) During fever therapy, of eighteen patients studied, fifteen, or 83 per cent, had abnormal values with ten ranging between 12 and 40 per cent. Following fever therapy fifteen of these patients had normal values.

index and serum bilirubin concentrations were determined. In the week prior to the appearance of the first positive smear for malaria, ninety-two tests were made on the group and all were negative. In a daily study for seven days from the finding of the first positive smear, there was an increase in positive tests from 6 to 41 per cent. This value was not exceeded in the seven days prior to termination of quinaerine therapy, and during six days of such treatment the positive values fell gradually to 6 per cent.

Serial Tests of Liver Function With Clinical Correlation.—It was not possible to carry out all the tests of liver function in all 138 patients before, during, and after fever therapy. However, fairly complete studies were made in thirty-two patients. The results of these studies are shown in Table IV, together with certain clinical findings in these cases.

All of the patients were considered to be good risks for fever therapy. None presented clinical evidence of hepatic disease prior to treatment, except that one patient showed a slightly, and another a moderately, elevated serum bilirubin (other tests in these patients were within normal limits).

Of twenty-nine patients adequately followed with liver function tests during the course of fever, twenty-one or 72 per cent showed results that deviated slightly or moderately from normal by one or more tests. Of these twenty-one patients sixteen showed hepatic enlargement, six showed hepatic tenderness, nineteen had nausea, thirteen had vomiting, and three developed clinical jaundice during the period of malarial activity. Two patients showed some hepatomegaly but no manifest alteration in liver function. In all instances except three (Patients 1, 13, and 32) the observed changes in liver function returned to normal after completion of malarial therapy. In only one of the three (Patient 32) was the residual abnormality such as to make the prognosis with respect to liver disease a guarded one.

At this hospital, of 172 patients treated with vivax malaria of foreign origin, eight men, or 5 per cent, developed clinical jaundice. In all eight cases the liver was enlarged and tender, bile was present in the urine, and the van den Bergh test was delayed or direct. The clinical picture was that of an acute hepatitis and not that of icterus caused solely by excessive hemolysis of red cells. Following fever therapy there was no clinical evidence of hepatic disease. In six of these patients on whom bromsulfalein tests were performed after fever therapy, the results were normal. In all instances termination of fever with quinaerine and the institution of general supportive measures (intravenous solutions of glucose, high carbohydrate and protein diets, and extra vitamins) resulted in a fairly prompt disappearance of the jaundice and a return of abnormal liver function tests to normal. In none was there evidence of permanent hepatic damage.

DISCUSSION

These patients with neurosyphilis formed a desirable group in which to study liver function because: (1) most of them had completed a standard course of chemotherapy; (2) an insectary for the raising of *Anopheles quadrimaculatus* mosquitoes for sporozoite inoculation was available; (3) it had

STUDIES OF PANCREATIC FUNCTION

III. THE EFFECT OF LIGATION OF THE PANCREATIC DUCTS UPON THE AMYLASE AND LIPIDASE CONTENT OF THE BLOOD

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IT IS well known and agreed that the effect of ligation of the pancreatic ducts is an immediate rise in the serum amylase and lipidase values.¹⁻⁴ There is less agreement, however, in the matter of the more chronic effects of ligation of the pancreatic ducts upon the concentration of these enzymes in the blood. Gould and Carlson¹ reported transient rises in the blood amylase for a period up to six weeks following pancreatic duct ligation. Golden and associates² demonstrated a permanent increase in blood amylase in three out of four dogs following the same operation. This is difficult to explain in view of their finding that the pancreas in all four dogs was converted into small, thin, cordlike remnants. Popper and Sorter³ kept two dogs alive for twelve months following pancreatic duct ligation. After a transient rise in the amylase at three weeks and six weeks postoperatively in one of the two animals, with a corresponding rise in the lipidase at three weeks, they found the amylase and lipidase values to be completely within the normal range for both dogs until the termination of the twelve months' period. Gross examination of the pancreas in both dogs showed it to be a thin, small, cordlike mass, while microscopic examination revealed an abundance of connective tissue with intact islets of Langerhans and small nests of cells which resembled acinar tissue. McCaughan⁴ approached this problem differently by introducing a rubber balloon into the duodenum and distending it with water until the pressure reached 90 mm. Hg; oxalated blood samples then showed a rise in amylase of 250 per cent in one animal and 380 per cent in another. However, after ligating the pancreatic ducts and allowing time for the pancreas to atrophy, repetition of occlusion of the pancreatic ducts by the distended balloon caused no rise in the blood amylase.

A somewhat similar situation occasionally occurs in patients following an attack of acute pancreatitis. Pinkham⁵ reported six cases in which there was persistent elevation of the serum amylase following the signs and symptoms of pancreatitis. Surgical exploration revealed the presence of collections of pancreatic fluid called pseudocysts, and drainage of these fluid collections promptly caused a decrease in the serum amylase in four of the six cases. Curiously enough, there was no quantitative relationship between the serum and the cyst fluid amylase values.

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(c) Following fever therapy sixty-six of the 138 patients were re-examined, and fifty-eight of them, or 88 per cent, were normal.

2. Cephalin flocculation tests using Wilson and Difco antigens showed that before inoculation and up until the first positive smear the percentage of 3 and 4 plus tests was zero; however, by the twenty-first day it was 100. No tests were performed after termination of fever.

3. Only one of 106 patients on whom galactose tolerance tests were performed prior to malarial therapy had an abnormal value. Following therapy thirty-one of these men were retested, and all had normal values.

4. The intravenous hippuric acid test was performed on twenty-five patients prior to fever therapy, and all results were normal. Twenty-two of these men were retested during fever therapy, and three had abnormal values. On the re-examination of two of them following therapy, one still remained abnormal. An additional ten of the original men were retested following fever therapy and gave normal findings.

5. The icterus index and serum bilirubin concentration were determined on the same sera of thirty-two patients. During the last seven days of fever therapy abnormal values were found in 38 and 17 per cent, respectively, with a rise to 47 and 24 per cent during the first four days of quinaerine treatment and a fall to 15 and 12 per cent in the next three days.

6. The urine urobilinogen test was performed on the same thirty-two patients just mentioned. All tests were normal prior to the first positive smear, but by seven days later 41 per cent were abnormal with a fall to 6 per cent abnormal tests at the end of quinaerine treatment.

CONCLUSIONS

1. Tests of liver function were carried out before, during, and after fever therapy in certain of 138 patients with neurosyphilis in whom malaria of Pacific or Mediterranean origin was induced by sporozoite or blood inoculation.

2. Although the results gave definite evidence of impairment of function in the majority of patients during fever therapy, serial studies during the two months following termination of fever with quinaerine showed a gradual return to normal, or to the condition prior to fever therapy, in practically all instances.

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Cat 1.—

Gross Findings: The pancreas appeared of normal length but was stringy and cordlike. No communication between the pancreas and the duodenum could be observed. A small pea-sized cyst filled with clear fluid was present on the exterior of the duodenum at the site of ligature, but no pancreatic tissue could be seen. The liver was of normal size but appeared yellowish and fatty.

Microscopic Examination: The pancreas was normal except for some eosinophilia of the distal cytoplasm of the acinar cells and some slight desquamation of the epithelium of a few of the alveoli. The islet and ductile tissue were normal. The liver showed small areas of intracellular fat infiltration and some abnormal dilation of the sinusoids.

Cat 2.—

Gross Findings: The pancreas appeared to be completely replaced by stringy, cordlike, fibrotic tissue. In the head of the pancreas near the second part of the duodenum a small hard mass which tapered off into the replaced pancreas could be palpated. On cutting into it, it was seen to be cystic, containing a clear watery fluid and continuous with the main pancreatic duct. No communications between the pancreas and duodenum could be demonstrated.

Microscopic Examination: The pancreas showed a marked increase in the interlobular connective tissue and a considerable reduction of acinar tissue. This was confined to small encapsulated areas among the extensive "scar" tissue. Most of the residual alveoli had dilated lumens lined with flattened epithelium. Rather extensive aggregations of lymphocytes had infiltrated into the hypertrophied connective tissue. Histiocytes and fibroblasts were numerous in these small round-cell clusters. There was no evidence of fat necrosis. The islet tissue was normal. The liver was normal except for an unusual number of von Kupffer cells.

Cat 3.—

Gross Findings: The pancreas appeared stringy and cordlike but of normal length, extending to the spleen. A small cyst, pinhead in size, was present at the site of ligature at the head of the pancreas.

Microscopic Examination: The pancreas had only a small amount of normal acinar tissue. Most of the alveoli showed a considerable amount of desquamation of the secretory epithelium. The lumens of some acini were dilated and the epithelium was flattened. The interlobular connective tissue showed slight hyperplasia. The islet and ductile tissues were normal. The liver was normal.

Cat 4.—

Gross Findings: The remnants of the pancreas were extremely difficult to recognize and identify as pancreas, since the area was almost completely fibrotic. A small cyst was present at the site of ligature at the head of the pancreas. No communication between the remnants of the pancreas and the duodenum could be found.

This paper is a report of work designed to obtain information upon the relationship of blood amylase and lipase to the atrophied, fibrotic pancreas. In another paper,⁴ it was demonstrated that following complete pancreatectomy in cats there was an initial decrease in both the serum amylase and lipase with a later irregular, but definite, return to control levels. A comparison of these results with the blood enzyme values in an animal with an atrophic pancreas, obtained by the same methods of analysis, was thought to be of interest.

EXPERIMENTAL

Four cats were anesthetized by intraperitoneal injection of nembutal, 30 mg. per kilogram of body weight. Control blood samples were obtained by heart puncture. After performing an upper midline incision, the pancreas was exposed and freed from the duodenum by blunt dissection. The area of the head of the pancreas containing the pancreatic ducts was doubly ligated with nonabsorbable sutures close to the ampulla of Vater and then cut between the ligatures. All pancreatic tissue remaining attached to the duodenum was carefully removed. The end of the pancreas was ligated again, and it was made certain that the pancreas was completely free of the intestine. The abdomen was closed with sutures and skin clamps. The animals were kept on a diet of dehydrated cat food, meat scraps from the hospital kitchen, and supplementary whole milk.

The determinations of serum amylase were carried out by the method of Somogyi,⁷ while serum lipase values were determined according to the method of Goldstein and Roe⁸ (tributyrin substrate).

RESULTS

The results are expressed graphically in Table I.

A description of the gross autopsy findings and the microscopic histologic features follows. All tissues were stained with Harris' hematoxylin and eosin and Mallory's triple connective tissue stains.

TABLE I

PERIOD AFTER LIGATION (MO.)	UNITS PER 100 C.C. OF SERUM							
	CAT 1		CAT 2		CAT 3		CAT 4	
	AMYLASE	LIPIDASE	AMYLASE	LIPIDASE	AMYLASE	LIPIDASE	AMYLASE	LIPIDASE
Control	1,767	130	590	137	910	142	925	131
1	701	112	330	59	756	106	34	144
2	840	128	157	128	810	109	953	117
3	1,109	104	635	78	889	83	908	90
4	822	110	928	119	888	109	950	144
5	772	82	759	135	698	92	1,495	92
8	1,891	148	1,155	178	1,153	149	907	147
Appearance of pancreas	Cystic		Cystic		Atrophic		Atrophic	
Gross	Normal		Incompletely		Incompletely		Completely	
Microscopic	areas		atrophic		atrophic		atrophic	

ducts, the serum amylase and lipidase concentrations returned to control levels in eight months postoperatively.

4. These data appear to show that the amylase and lipidase concentrations of the blood serum are markedly influenced by extra pancreatic factors.

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Microscopic Examination: The pancreas showed complete atrophy of the acinar tissue. Small nests of normal islet tissue were found in the markedly increased connective tissue septa. Some areas of lymphocytic infiltration were noted. A large dilated duct occupied a sizable portion of the atrophic gland. The liver was normal.

DISCUSSION

In a previous work⁴ we have demonstrated an immediate rise in serum amylase and lipase following pancreatic duct ligation. It was the purpose of these experiments to show later effects of ligation of the pancreatic ducts. At the one- and two-month intervals after ligation, all four animals showed amylase concentrations below the control values, and, in some instances, the values were remarkably subnormal. Following this, there was a gradual rise in amylase concentrations until the eighth month after ligation, when the amylase determinations had returned to the preoperative levels.

The lipase determinations were less clear-cut, but in the first three months postoperatively there was a recognizable decrease in the serum lipase values. Following this period, there was a gradual rise in the lipase concentrations until the eighth month after ligation, when the lipase values were essentially the same as the control values.

It is of considerable interest that in the microscopic examination one pancreas showed normal areas of acinar tissue, two showed considerable necrosis with little recognizable acinar tissue, and the fourth was completely atrophic. In spite of the variations in the microscopic picture, the serum amylase and lipase of all four animals show the same postoperative trends. The obvious conclusion is that subsequent return to control levels was due to extra pancreatic factors.

If the conclusions from these laboratory experiments can be translated to clinical laboratory findings, it follows that the serum lipase determination is as nonspecific as the amylase test in chronic pancreatic disease.

These data support the same conclusions arrived at by total pancreatectomy experiments, namely, that there are other sources of amylase and lipase in the animal body which may operate to produce as high a level of these enzymes in the blood as when a normally functioning pancreas is present.

SUMMARY

1. Studies of the amylase and lipase content of the blood serum of cats following ligation of the pancreatic ducts over a period of eight months have been made.

2. Serum amylase and lipase concentrations, which previous work has shown are at first rapidly increased by ligation of the pancreatic ducts, showed marked decreases in from one to three months after ligation.

3. In one animal with a completely atrophic pancreas and in three others with varying degrees of pancreatic necrosis due to ligation of the pancreatic

rubber stopper having a cutout through which the animal's tail protruded. With the rat inside, this carton was placed on the heating coil of a 56° C. incubator and was heated for a minute or longer in order to induce dilatation of the tail veins. When the heating was adequate, the tail veins were distinctly visible. When slight pressure was applied with a finger over the base of the tail, the distal portion of the vein became distended with blood and could be easily tapped with a No. 26 hypodermic needle. One should be able to draw blood into the syringe both before and after the completion of the injection. Once the technique of intravenous injection is mastered, rats can be injected at the rate of thirty per hour.

Alloxan monohydrate was prepared as a 2 to 5 per cent aqueous solution and was injected in various doses from 15 to 200 mg. per kilogram intravenously and from 50 to 200 mg. per kilogram intraperitoneally. Serial blood sugars were determined by the Folin-Malmros micro method (1929)⁶ at 0, 3, 8, 24, 48, and, at times, 72 hours. Nonprotein nitrogen determinations were carried out on the tungstate filtrates obtained in the Folin-Malmros procedure. The filtrates were charred and microdistilled, and the nitrogen was determined by direct nesslerization, using Koch's modification (1941)⁷ of the Nessler-Folin reagent. One-tenth of a cubic centimeter of blood obtained by cutting the tip of the tail sufficed for both the blood sugar and the N.P.N. determinations. Following the last sugar determination the animals were killed by a blow on the head, perfused through the aorta with a small amount of saline, followed by about 200 c.c. of formol-Zenker solution. Tissues were immersed from six to ten hours in formol-Zenker solution; then they were washed, dehydrated, and embedded in paraffin according to the usual methods. Sections of the pancreas, kidney, liver, and adrenal were stained with hematoxylin-eosin. The pancreas was also stained by a slight modification of Bensley's neutral crystal violet method (Palay and Lazarow, 1946⁸). Selected sections of liver were stained by Wilder's stain for reticulum.

RESULTS

Blood Sugar Response.—Following the injection of alloxan three phases in the blood sugar response were noted: an initial hyperglycemia, followed by a transient hypoglycemia, and finally, a late stage of permanent hyperglycemia or diabetes. The durations of these phases were somewhat variable from animal to animal. The blood sugar values of normal rats allowed excess food and water practically always fell within the range of 90 to 160 mg. per 100 c.c.

In Fig. 1 is shown the blood sugar response of thirty-six rats given alloxan intravenously in doses of 40 mg. per kilogram. The blood sugar value is designated by the height of the rectangle. At three hours only hyperglycemic reactions were noted. Hypoglycemic reactions appeared at twenty-four hours and a few at forty-eight hours. Many hypoglycemic reactions were probably missed because of the limited number of blood sugar determinations. By forty-eight hours, however, most of the animals had reached their permanent hyperglycemic phase or diabetes.

THE PRODUCTION AND COURSE OF ALLOXAN DIABETES IN THE RAT

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IT WAS usually noted in preliminary studies on the rat that when alloxan alone was given in doses of 200 mg. per kilogram more than 80 per cent of the animals developed diabetes (Lazarow, 1945¹). Occasionally, however, a group of animals failed to respond to this dose of alloxan. Because it is desirable to be able to produce diabetes consistently in the rat, the present study was undertaken.

Numerous investigators have studied alloxan diabetes in the rat. Although all have used parenteral routes, there is disagreement as to the best method of producing diabetes. Dunn and McLetchie (1943)² used repeated subcutaneous injections. Gomori and Goldner (1943)³ reported consistent results using one intraperitoneal injection of alloxan (200 mg. per kilogram) and stated that subcutaneous injections of 50 to 175 mg. per kilogram were not effective. Bailey, Bailey, and Leech (1944),⁴ however, reported that intraperitoneal and intravenous injections of alloxan in the rat were not satisfactory, whereas a subcutaneous dose of 200 mg. per kilogram did produce diabetes regularly. Gomori and Goldner also reported that hooded rats were resistant to alloxan. But Duff and Star (1944)⁵ stated that if hooded rats are injected with larger doses (from 175 to 350 mg. per kilogram) of alloxan they, too, develop diabetes.

Since alloxan is rapidly destroyed in body fluids (or in the blood), it seems that theoretically, at least, the intravenous route of administration would be preferable. This is actually the case, for the intravenous method avoids the highly variable factor of destruction which occurs during alloxan absorption, and hence, diabetes is produced more consistently. The amount of alloxan needed to produce a given level of diabetes is but a small fraction of that required when the alloxan is administered by a nonintravenous route. This paper compares the diabetes produced in the rat by alloxan administered intravenously with that produced by intraperitoneal injection.

METHODS

Sprague-Dawley male and female albino rats were used in this study and were allowed excess food and water. For the intravenous injection rats were immobilized by placing them in a cylindric mailing carton of appropriate size. This carton was provided with air holes at one end and at the other end with a

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considered as showing no response to alloxan. As a general rule the level of the blood sugar at forty-eight hours was a measure of the severity of the diabetes.

Relation of Dose of Alloxan to Blood Sugar Response.—The incidence of blood sugar responses as well as the average forty-eight hour blood sugar in each group is given in Table I. The forty-eight hour average includes the blood sugars of those animals which did not show any response to alloxan.

TABLE I. COMPARISON OF EFFECTS OF GRADED DOSES OF ALLOXAN INJECTED INTRAVENOUSLY AND INTRAPERITONEALLY

DOSE ALLOXAN (MG. PER KG.)	TOTAL NUMBER OF ANIMALS	AVERAGE INITIAL WEIGHT (GM.)	PER CENT WEIGHT LOSS (AT 48 HOURS)	PER CENT ANIMALS WITH BLOOD SUGAR RESPONSE	AVERAGE 48-HOUR BLOOD SUGAR	PANCREATIC DAMAGE (NUMBER OF ANIMALS)				BLOOD N.P.N. (NUMBER OF ANIMALS)				KIDNEY TUBULE NECROSIS (NUMBER OF ANIMALS)				LIVER NECROSIS (NUMBER OF ANIMALS)				
						SEVERE	MODERATE	MINIMAL	NORMAL	AVERAGE (AT 48 HOURS)	INCREASE (50% OR LESS)	INCREASE (50 TO 150%)	INCREASE (150 TO 350%)	INCREASE (350% OR MORE)	NORMAL	SLIGHT	MODERATE	SEVERE	NORMAL	SLIGHT	MODERATE	SEVERE
Intravenous																						
100	2	150	13	100	-	2	0	0	0	508	0	0	0	2	0	0	0	2	2	0	0	0
75	2	171	15	100	933	2	0	0	0	223	1	0	0	1	2	0	0	0	1	1	0	0
50	8	186	10	100	509	8	0	0	0	182	4	2	0	2	3	4	1	0	6	0	0	2
40	18	204	10	94	333	17	1	0	0	42	17	0	1	0	16	2	0	0	6	4	3	5
30	4	192	3	75	346	2	2	0	0	67	3	1	0	0	3	1	0	0	2	0	0	2
25	2	230	5	50	239	1	1	0	0	46	2	0	0	0	2	0	0	0	2	0	0	0
20	4	146	0	25	189	1	1	0	2	47	4	0	0	0	3	1	0	0	2	0	1	1
15	2	220	0	0	126	0	1	0	1	41	2	0	0	0	2	0	0	0	2	0	0	0
Intraperitoneal																						
200	8	241	12	75	366	6	2	0	0	133	1	3	1	3	0	3	2	3	3	4	0	1
150	3	307	6	66	396	2	0	0	1	66	1	2	0	0	1	2	0	0	0	2	1	0
100	4	184	3	0	116	0	1	2	1	52	4	0	0	0	3	1	0	0	3	1	0	0
50	4	159	0	0	109	0	0	1	3	39	4	0	0	0	4	0	0	0	3	1	0	0

It is clear that when alloxan was administered intravenously in doses of 50 mg. per kilogram or greater all of the rats responded. After a dose of 40 mg. per kilogram intravenously seventeen of eighteen rats responded. In sixteen additional rats given 40 mg. per kilogram of alloxan intravenously and not included in Table I, all showed a blood sugar response. As the alloxan dose was reduced, the incidence of blood sugar response decreased, and at a dose of 15 mg. per kilogram intravenously none showed any blood sugar response. In contrast to these results following intravenous administration, when 200 mg. per kilogram were given intraperitoneally, only six of eight animals showed a blood sugar response, and after a dose of 100 mg. per kilogram intraperitoneally, none showed any blood sugar response. Thus 40 mg. per kilogram of alloxan administered intravenously produced a more consistent effect than did 200 mg. per kilogram administered intraperitoneally.

severe, all animals showed the typical blood sugar response and developed diabetes. However, the severity of the diabetes as judged by the forty-eight hour blood sugar could not be predicted by studying the extent of cytologic damage in the pancreas. Foglia (1944)¹⁰ observed that after removal of 95 per cent of the pancreas rats developed manifest diabetes only after several months. The progression of the disease in such partially pancreatectomized animals may be attributed to degeneration of the remaining beta cells (Allen, 1913¹¹; Homans, 1913, 1914¹²). Therefore, it is reasonable to suppose that in order to produce immediate diabetes in the rat by alloxan, it is probably necessary to destroy or inactivate more than 95 per cent of the beta cells. Probably one cannot expect to recognize by histologic examination slight increments in the extent of beta cell damage beyond 95 per cent destruction. As the dose of alloxan was reduced, however, many of the animals which showed moderate changes in the pancreas (that is, degranulation or necrosis of only some of the beta cells) did not develop diabetes and showed only very slight, if any, fluctuations in their blood sugars. Thus, cytologic examination of the pancreas will detect moderate damage of the beta cells more accurately than will a study of the blood sugar response.

Renal Damage.—In addition to the pancreatic lesions, alloxan also produces renal damage. The tubules are usually involved, and the severity of the injury varies from hydropic degeneration to necrosis (Bailey, Bailey, and Leech, 1944⁴; Dunn and McLetchie, 1943²). In our series necrosis of the tubules was commonly focal, but when extensive it involved all lobules of the kidney. The affected tubules usually were filled with necrotic cells and debris. Often all the epithelial cells had desquamated into the lumen. In some cases, however, there were many young epithelial cells containing basophil cytoplasm and nuclei in mitosis, an indication that repair had begun. Infiltration of polymorphonuclear leucocytes around the affected tubules occurred in only a few specimens.

In Table I the extent of tubular necrosis is listed. A kidney showing only occasional isolated small foci of tubular necrosis was considered to be slightly damaged. A kidney with many isolated necrotic areas was considered to be moderately damaged, and one in which the areas of necrosis were large and confluent was considered to be severely damaged. Hydropic degeneration and cloudy swelling are not listed in the accompanying table, because the extent of this type of damage was more difficult to classify than was necrosis.

Both the incidence and severity of the renal lesions were directly related to the dose of alloxan administered. At 40 mg. per kilogram intravenously sixteen of eighteen rats showed no significant changes, whereas two showed only slight changes in the kidney tubules. In contrast, at 200 per kilogram intraperitoneally, none of the rats were entirely normal, and more than half of the animals showed moderate to severe changes in the kidney. Thus, although the intravenous administration of 40 mg. per kilogram of alloxan was as effective in producing diabetes as was the larger intraperitoneal dose of 200 mg. per kilogram, the incidence of lesions in the kidney was markedly reduced by giving the smaller dose intravenously.

The severity of the blood sugar response is directly related to the dosage of alloxan administered. Thus, after doses of 75 to 200 mg. per kilogram intravenously, many of the forty-eight hour blood sugar values were in the neighborhood of 1,000 mg. per cent. As the alloxan dose was reduced, the blood sugar at forty-eight hours was also less elevated. At 40 mg. per kilogram intravenously most of the forty-eight hour blood sugars fell between 200 and 500 mg. per cent, and in only one animal out of thirty-four was it over 600 mg. per cent. At still lower dosages those animals which reacted to alloxan usually showed a forty-eight hour blood sugar of 300 to 400 mg. per cent. The average forty-eight hour blood sugar value, however, given in Table I is weighted by the inclusion of the animals that did not react.

When doses of 200 mg. per kilogram of alloxan were injected intraperitoneally, the average forty-eight hour blood sugar was about the same as that observed following an intravenous dose of 40 mg. per kilogram. Although the group of rats which received 200 mg. per kilogram intraperitoneally was smaller than the group which received 40 mg. per kilogram intravenously, the distribution of the forty-eight hour blood sugars of the diabetic animals in the two groups was the same. Thus, the severity of the diabetes produced by alloxan injected intravenously in doses of 40 mg. per kilogram was of about the same degree as that produced by the intraperitoneal injection of alloxan in doses of 200 mg. per kilogram.

Weight Loss.—Animals which showed a blood sugar response following the injection of alloxan also showed considerable weight loss in forty-eight hours, usually about 10 per cent of their body weight.

Pancreatic Damage.—The morphologic changes produced by alloxan in the islets of Langerhans have recently been reviewed by Duff (1945).⁹ These changes consist mainly of selective necrosis and disappearance of the beta cells with consequent destruction of the architecture of the islets. The beta cells undergo degranulation, shrinkage, and nuclear pyknosis and are finally removed from the islet. The completely affected islet typically is composed of a cluster of capillaries surrounded by a band of normal alpha cells. The absence of inflammatory reaction is striking.

In Table I the degree of damage to the islets has been classified into three main groups: severe, moderate, and minimal.

In islets which were included in the first group, the beta cells had either completely disappeared or were all shrunken and detached and contained pyknotic nuclei. Islets which contained necrotic beta cells and a moderate number of normal beta cells were considered as moderately damaged. Included in the same group were islets in which most of the beta cells were simply degranulated but still contained normal nuclei. When the architecture of the islets was intact and most of the beta cells were normal (a few necrotic or degranulated), the islets were considered to show only minimal changes.

In general, there was very good correlation between the histologic findings and the blood sugar response (Table I). When the pancreatic damage was

In Table I the necrosis of the liver following intravenous injection of alloxan has been classified according to the extent of involved tissue. When only occasional foci of necrosis appeared in the cross sections of the entire liver, the damage was considered slight. In moderately affected livers there was approximately one focus of necrosis in the area of three or four lobules. Severely affected livers had at least one focus of necrosis in each lobule.

When alloxan was given intraperitoneally, the necrosis, although focal, was not dispersed throughout the organ. The lesions were principally subcapsular and limited to the free edge of the lobes, and the subdiaphragmatic portions of the liver suffered little or no damage. The necrosis often involved a band only two or three cells deep under the peritoneum, but in a few specimens the necrosis extended deep into the liver substance. In only one rat was the damage sufficient to be called severe.

The incidence and severity of the hepatic involvement cannot be adequately correlated with either the dosage of alloxan or the incidence of renal lesions. Some of the highest intravenous alloxan doses did not produce hepatic lesions, whereas some of the lowest were associated with severe damage. In some of the animals which showed extensive renal involvement, the livers were normal; whereas in others severe hepatic necrosis was not associated with renal change. Very occasionally after administration of a nondiabetogenic dose of alloxan, some hepatic involvement was noted in the absence of any pancreatic lesions.

Course of Diabetes Produced by Injection of 40 Mg. Per Kilogram of Alloxan Intravenously.—Sixteen rats were given alloxan in doses of 40 mg. per kilogram intravenously and were followed serially for several months (Table II). They were kept on a diet of Friskies dog food* and water. The blood sugars, weights, and water intakes were determined daily. All sixteen rats responded to alloxan.

TABLE II. ALLOXAN DIABETES IN THE RAT (40 MG. PER KILOGRAM INJECTED INTRAVENOUSLY)

RAT	INITIAL WEIGHT	AVERAGE BLOOD SUGAR VALUE							
		DAYS SURVIVAL	FIRST WEEK	SECOND WEEK	THIRD WEEK	FOURTH WEEK	FIFTH WEEK	SIXTH WEEK	SEVENTH WEEK
8	278	Alive	291	136	123	132	122	132	134
6	274	Alive	339	191	247	286	382	369	402
5	287	Alive	353	258	322	396	378	366	389
3	293	Alive	378	409	426	438	450	430	416
13	288	Alive	337	332	408	404	458	450	498
14	317	Alive	368	406	429	482	486	466	491
1	258	Alive	444	417	430	599	565	505	544
11	330	Alive	352	403	424	506	516	564	540
15	298	29	409	436	488	542	-	-	-
2	292	23	456	451	582	704	-	-	-
7	264	4	355	-	-	-	-	-	-
4	228	4	504	-	-	-	-	-	-
16	290	4	360	-	-	-	-	-	-
17	284	4	578	-	-	-	-	-	-
18	299	5	358	-	-	-	-	-	-
12	309	6	406	-	-	-	-	-	-

*Albers Milling Co.

Blood Nonprotein Nitrogen.—In Table I is listed the average forty-eight hour N.P.N. and also the degree of elevation in N.P.N. for the various animals within the group. Fluctuations with ± 50 per cent of the initial N.P.N. were considered within the normal range, for the animals were not fasted and control animals sometimes showed this much fluctuation. Again, when the effect of 40 mg. per kilogram intravenously is compared with that produced by 200 mg. per kilogram intraperitoneally, it is noted that seventeen of eighteen animals given the lower dose intravenously showed no significant elevation in the N.P.N. Only one animal showed an increase in its N.P.N. value of 200 per cent. In contrast, however, after the larger dose intraperitoneally, four of eight animals showed an increase of 300 per cent or greater, and three showed an increase in N.P.N. of 50 to 150 per cent. Only one of the eight animals in this latter group showed no significant change.

In general, the elevation in the nonprotein nitrogen of the blood following alloxan injection paralleled the severity of the renal damage observed histologically. In a few instances there was evidence of slight kidney necrosis without any significant rise in the N.P.N. In three cases which had received 40, 50, or 75 mg. per kilogram of alloxan intravenously, the forty-eight hour N.P.N. was markedly increased, from 113 to 399 mg. per cent, and yet the kidney exhibited only slight evidence of necrosis. The tubules, however, did show considerable hydropic degeneration. Two of these animals were also unusual in that they passed through a delayed hypoglycemic phase at forty-eight hours, and all showed a very intense hyperglycemia at some time during the forty-eight hours, from 629 to 1244 mg. per cent. How much of the azotemia was due to renal and how much to extrarenal factors is difficult to determine. However, since uric acid values up to 100 mg. per cent have been reported in the pigeon following alloxan administration (Goldner and Gomori, 1945¹³), part of the elevation in N.P.N. might be due to this nitrogenous substance.

Liver Damage.—The typical lesion produced in the liver by intravenous injection of alloxan was a small circular focus of necrosis usually located in the midzonal or peripheral region of the lobule. The lesions, which varied in diameter from 45 to 200 micra, were usually smaller than 100 micra and were distributed uniformly throughout all lobes of the liver. Intact liver cells were usually completely absent from the focus, and the sinusoids were collapsed or distorted. The normal reticular framework was fragmented or absent. The center of the lesion was occupied by large mononuclear cells which appear similar to the Kupffer cells in the surrounding intact parenchyma. These cells frequently contained fragments of pyknotic nuclei or hyalin acidophil masses. Inflammatory reaction was usually slight or absent. Shrunken acidophil remnants of liver cells were scattered throughout the lesion and at the margins. The liver cells in the surrounding cords were either entirely normal or contained large cytoplasmic vacuoles and hyperchromatic nuclei. When the liver showed extensive necrosis, the remaining parenchyma usually showed fatty metamorphosis; however, in livers with only occasional foci of necrosis, the rest of the organ was usually normal.

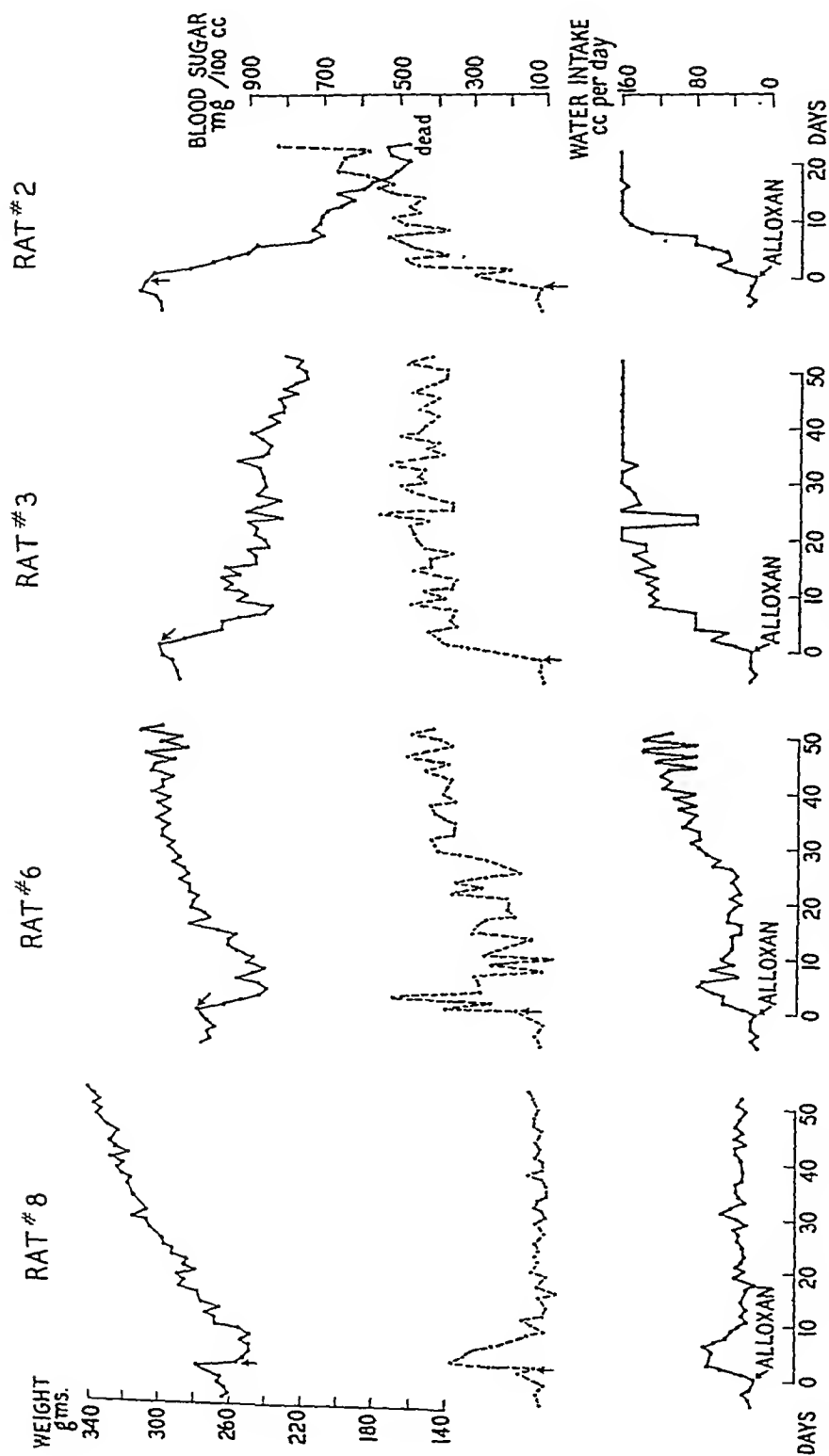


Fig. 2.—Course of alloxan diabetes in the rat (four selected cases). Alloxan was given in doses of 10 mg. per kilogram intravenously. The weights, blood sugar, and water intakes were measured daily.

Five animals died between the fourth and sixth days with terminal blood sugars ranging from 360 to 560 mg. per cent. One of the animals showed a significant increase in the blood N.P.N. at forty-eight to seventy-two hours; the others were normal at this time. A sixth animal (Rat 16), although it had a blood sugar of 475 mg. per cent at forty-eight hours, died on the fourth day with a blood sugar of only 190 mg. per cent just prior to death. All of the remaining ten rats survived many weeks. Of the surviving animals one (Rat 8) showed transitory diabetes lasting for five or six days (Fig. 2). After this period the blood sugar returned to normal, the weight started to increase, and the water intake fell off to normal. A second animal (Rat 6), whose blood sugar at forty-eight hours was 530 mg. per cent, showed wide fluctuations in the daily blood sugar which frequently fell below 200 mg. per cent. However, as time progressed the average blood sugar tended to rise, fluctuations below 200 mg. per cent occurred less frequently, and finally, after thirty-six days the blood sugar tended to stabilize at about 370 to 400 mg. per cent. During the period of progressive rise in blood sugar the water intake increased, and the weight gain was less rapid than that occurring in the early stages of the diabetes.

During the first several weeks following the onset of the diabetes most of the eight remaining rats showed a slight but progressive increase in their average blood sugars (Table II). For example, the average blood sugar of Rat 15 increased from 409 mg. per cent during the first week to 436 the second, 488 the third, and 542 the fourth. Rat 2 which had an average sugar of 456 during the first week died on the twenty-third day with a terminal sugar of 824 mg. per cent.

In Fig. 2 is shown the course of the diabetes for selected animals from this group. The weight curves of these animals depended upon the severity of the diabetes. When the blood sugar value was moderately elevated, the weight tended to be maintained (Rat 6). With higher blood sugar values the rats tended to lose weight progressively (Rats 2 and 3). Some of these diabetic rats drank more than their own weight of water within twenty-four hours.

DISCUSSION

It is clear from these studies that the intravenous administration of alloxan in doses of 40 mg. per kilogram is a satisfactory method for the production of diabetes in the rat. Alloxan is an unstable compound which is destroyed rapidly at pH 7.4. It has been reported that it disappears from the blood within two minutes after its injection (Leech and Bailey, 1945¹⁴). Consequently it is not surprising that the dose of alloxan required to produce diabetes by the intraperitoneal route is much larger than that required by the intravenous route. Variations of the absorption rates of alloxan coupled with its rapid destruction during absorption could account for the inconsistencies reported in the literature. The intravenous method, however, eliminates these variable factors. It should be emphasized that the alloxan must be injected as rapidly as possible because during slow injection there may be increased destruction. Lower doses for intravenous injections have been used in the dog (Goldner and Gomori, 1943¹⁵).

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It is interesting that some animals given 40 mg. per kilogram of alloxan intravenously showed a progressive increase in the severity of their diabetes as judged by the average blood sugar. After partial pancreatectomy rats may show a period of obesity without diabetes, followed by a period of latent diabetes, and finally, a period of manifest diabetes (Foglia, 1944¹⁰). Thus the beta cells remaining, although adequate to meet the body's needs for a time, later are unable to secrete sufficient amounts of insulin, and hence, the animal develops diabetes. Overwork and exhaustion of the beta cells have been given as explanations for this progression (Allen, 1913¹¹; Homans, 1913, 1914¹²). Following a dose of alloxan of 40 mg. per kilogram most of the beta cells are destroyed. But it is likely that a few functional beta cells still remain, for when larger doses of alloxan are given intravenously (that is, from 75 to 100 mg. per kilogram), the diabetes which develops within forty-eight hours is more severe. Therefore, the progressive rise in the blood sugar observed over a period of weeks in rats given only 40 mg. per kilogram of alloxan may result from the exhaustion of the few remaining beta cells.

SUMMARY

1. The rapid intravenous injection of alloxan is more satisfactory for producing diabetes in the rat than is intraperitoneal injection.

(a) When alloxan is injected intravenously only a small dose is required, and the incidence and severity of the resulting diabetes are more uniform than when five times that dose is given intraperitoneally.

(b) By the intravenous injection of 40 mg. per kilogram of alloxan it is possible to produce diabetes in most rats without significant renal damage.

2. Sixteen rats were given 40 mg. per kilogram of alloxan intravenously, and their blood sugars, weights, and water intakes were followed for many weeks.

(a) Of these sixteen rats five died within the first four to six days with terminal blood sugars ranging from 360 to 560 mg. per cent.

(b) Ten of the sixteen rats survived many weeks.

(c) One which developed transitory diabetes during the first week recovered and remained normal for the following six weeks.

(d) During the first few weeks following the onset of diabetes, the average blood sugar levels of most of the surviving rats showed a slight but progressive rise and usually attained values of 400 to 600 mg. per cent.

(e) Depending on the severity of the diabetes the animals gained weight very slowly or progressively lost weight.

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More simply, the proportion of foreign blood ($\frac{v}{V}$) is $1 - e^{-t}$ if t is expressed in units of T . In Fig. 1 this is shown in graphic form.

By substituting numeric values for t , the data in Table I are obtained.

For practical purposes the blood volume of a newborn infant can be considered to be about 250 c.c., so little is gained by the transfusion of more than 500 c.c. of blood. As Wallerstein⁸ has pointed out, it is advantageous to inject from 50 to 100 c.c. more blood than has been withdrawn, which serves further to increase the proportion of donor's blood.

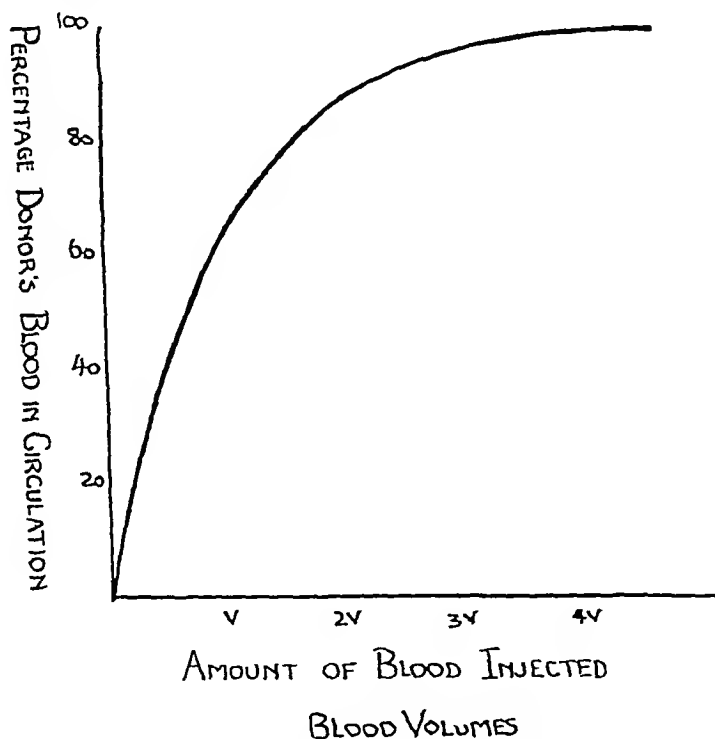


Fig. 1.—Percentage of donor's blood in patient's circulation after infusion and withdrawal of varying volumes of blood. In this figure, V represents an amount of blood equal to the patient's blood volume.

TECHNIQUE

The main obstacle to the successful performance of an exchange transfusion in infants is not the injection but the process of withdrawal, which is hampered by the coagulation of the infant's blood. This is circumvented by the use of heparin.

To test the efficacy of the procedure, a Mongolian idiot, 28 days old, weighing 7 pounds, was used. The infant belonged to group $A_2MNRh_1Rh_2$. A sample of bank blood drawn into sodium-citrate-citric-acid-dextrose mixture four days previously, belonging to group $A_1MRh_1Rh_2$ and compatible on cross-match, was selected for the transfusion. The cells had sedimented, and we removed about

THE USE OF HEPARIN WHEN PERFORMING EXCHANGE BLOOD TRANSFUSIONS IN NEWBORN INFANTS

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AT LEAST three distinct clinical entities are grouped under the term erythroblastosis fetalis.^{1,2} The importance of recognizing the differences between these entities is that the treatment and prognosis vary with the manifestation. Those cases that are characterized by anemia with slight jaundice (congenital hemolytic disease) respond readily to proper transfusion therapy, while infants who show severe jaundice with little or no anemia (icterus gravis) frequently die within a short time after the onset of the disease, usually with the post-mortem finding of kernicterus.⁴ Of this latter group some survive but may later develop sequelae of severe cerebral damage or hepatic cirrhosis.⁵

The fact that the disease usually has its onset at birth or very shortly thereafter offers the hope that if a reasonably accurate prediction can be made during pregnancy concerning the likelihood of the disease occurring, as well as the type of manifestation, the lives of these babies can be saved and the sequelae prevented. The newer tests for the Rh-Irr types, as well as the development of methods of detecting the presence or absence of sensitization, now offer the prospect of more accurate forecasting.^{6,7} If, as has been proposed by one of us, the syndrome of icterus gravis is initiated by intravascular agglutination or coagulation,^{1,2} then exchange transfusion with blood completely compatible with the maternal serum should effectively prevent the onset of the disease process or arrest it after it has begun.

The purpose of this paper is to describe a technique of performing exchange transfusions in newborn infants, which has been successful in our hands.

THEORETIC CONSIDERATIONS

If the infant is to survive the procedure, blood must be injected and withdrawn simultaneously and at approximately equal rates.

Let us assume that in an infant with the blood volume V the speeds of withdrawal and injection of blood are uniform and equal. Let T represent the time required to withdraw and inject a quantity of blood equal to V and t the time to inject and withdraw any given volume of blood, while v represents the volume of donor's blood in the patient's circulation at time t . Then:

$$\text{When } t = 0, v = 0 \text{ and } \frac{dv}{dt} = \frac{V}{T}$$

$$\text{When } t = \infty, v = V \text{ and } \frac{dv}{dt} = 0$$

$$\text{Therefore, } \frac{dv}{dt} = \frac{V - v}{T}$$

$$\text{Hence, } v = V (1 - e^{-\frac{t}{T}})$$

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when kept overnight. The occurrence of clotting in the last tube was probably due to the washing out of the heparin from the circulation by the infused blood. It appears that the amount of heparin used is innocuous, because in a previous test designed to determine a safe heparin dosage for the procedure, quantities as high as 5 c.c., or five times the amount used in the transfusion, were given intravenously without harmful effects, even though the infant's blood remained incoagulable for thirty-six hours.

The entire procedure took only ninety minutes and was carried out with facility. This contrasts sharply with our previous experience¹⁰ without the aid of heparin, when only with considerable difficulty, and by tapping numerous vessels including the radial artery, did we succeed in withdrawing the required amount of blood after three hours.

SUMMARY

A simple technique, involving the use of heparin, is described for performing complete exchange transfusions in newborn infants, a procedure which may be indicated in severe cases of icterus gravis.

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TABLE I.

AMOUNT OF BLOOD INJECTED	PERCENTAGE OF DONOR BLOOD IN BODY
$\frac{1}{2}$ Blood volume	39.4
1 Blood volume	63.2
$1\frac{1}{2}$ Blood volumes	77.7
2 Blood volumes	86.5
$2\frac{1}{2}$ Blood volumes	91.8
3 Blood volumes	95.0

three fourths of the plasma and made the total volume up to 500 c.e. again with sterile saline solution. The internal saphenous vein was exposed at the left ankle, and a blunt 20 gauge cannula was tied in place with catgut. One-half cubic centimeter of Upjohn's heparin, equivalent to 500 units, was injected through the needle, and the gravity apparatus containing the blood was connected. There was considerable oozing at the site of the incision, but this was readily controlled by pressure. An incision was then made at the right wrist and a short bevel 20 gauge needle inserted into the radial artery. By this time the infant had received about 50 c.e. of blood through the drip infusion. The infant's blood flowed freely from the radial puncture and was collected fractionally for differential agglutination studies. The speed of the infusion was regulated constantly to keep pace with the radial flow and to maintain the original 50 c.e. advantage.

When 150 c.e. of blood had been withdrawn, some clotting occurred in the radial cannula; therefore, another 0.5 c.e. of heparin was administered, this time into the radial artery. Except for one short period when the infant became pale and showed evidence of air hunger, the procedure went quite smoothly. The infant's color and normal respirations were re-established by increasing the rate of flow of the infusion. After 400 c.e. of blood had been withdrawn, the radial artery was ligated and the skin sutured. The infusion was permitted to continue until the entire 500 c.e. of blood were administered. The saphenous vein was then ligated and the skin sutured. Only slight oozing occurred, and this was readily controlled by pressure bandages. The infant's condition at the end of the procedure was excellent. A blood count done on the following day showed a hemoglobin concentration of 88 per cent* and red cell count of 4.2 million per cubic millimeter. A blood count taken two weeks prior to the procedure showed a hemoglobin concentration of 120 per cent and red cell count of 4.7 million. The larger drop in hemoglobin concentration in comparison with the drop in the red cell count is due to the substitution of normocytic cells for the macrocytic cells which characterize the neonatal period.

The efficacy of the procedure was demonstrated by the differential agglutination test⁹ done on the samples of blood withdrawn from the infant at various stages of the procedures. The increase in the proportion of donor's cells roughly followed the pattern shown in Fig. 1, and the last sample of blood showed that even before the final 50 c.e. had been injected approximately 90 per cent of the red cells in the infant's circulation were the donor's. Incidentally, the blood samples in all the tubes except the last failed to clot even

*One hundred per cent equivalent to 15 Gm. per 100 c.c.

only agglutinins (bivalent antibodies), not glutinins (univalent antibodies), because the latter occur only in immune sera. Material suitable for testing the hypothesis could only be obtained from cases of erythroblastosis fetalis caused by A-B sensitization.¹⁰ The purpose of this report is to present the results of alpha and beta antibody studies on the mothers and infants of two such cases.

CASE REPORTS

CASE 1.—The clinical details of this case have been described in full in another paper.¹⁰ The infant, a first-born male infant, developed deep jaundice and mild anemia immediately after birth. The jaundice disappeared within ten days, and the infant recovered spontaneously and completely. This is the typical story of mild erythroblastosis due to A-B sensitization (icterus precox).^{10, 11} The father of the patient belonged to group A, MNRh,rh , the mother to group OMNRh, Rh_1 , and the patient to group A, MNRh,Rh_1 .

CASE 2.—The patient, a male infant, was the third child. The first child, born seven years previously, developed severe erythroblastosis within twenty-four hours after birth. It was treated first by intramuscular injections of maternal serum (the worst possible treatment according to present knowledge but a treatment which had been recommended for this condition in 1939). Thereafter the infant was given repeated transfusions of 75 to 100 c.c. of whole citrated blood (fourteen transfusions in all) but finally died at the age of four months, still deeply jaundiced. The second infant, born in 1941, was apparently normal at birth and was given 50 c.c. of maternal serum intramuscularly, presumably for purposes of prophylaxis. Shortly after this the infant developed jaundice and anemia. At this time the role of the Rh factor in erythroblastosis had just been discovered, and the child was transfused empirically with Group O, Rh-negative blood. Complete recovery followed three such transfusions of a total 200 c.c. of blood. The grouping tests on the parents and the living child were as follows: father, A, MNRh,Rh_2 ; mother, OMNRh, Rh_2 ; child, A, MRh,Rh_1 . These findings and titrations of the alpha and beta antibodies in the maternal serum indicated that sensitization of the mother to the A factor, and not to the Rh factor, was responsible for the occurrence of erythroblastosis in the first two children of this family. The second infant recovered not because the blood donor used was Rh negative but because he belonged to Group O. On the other hand, the first infant died probably because of the persistent use of homologous Group A blood. It was anticipated before the birth of the third child, our patient, that this infant also would be erythroblastic. Therefore, immediately after delivery, the cord was clamped and 10 c.c. of Witebsky's solution of A and B group substances were injected into the infant through the vessels of the umbilical cord. As expected, the infant proved to be jaundiced, and the blood smear showed as many as ten normoblasts and erythroblasts per oil immersion field. A mild anemia developed on the second day, and the infant, who weighed only 5 pounds, was given a transfusion of the washed red cells from 50 c.c. of the mother's blood together with 10 c.c. of the A and B group substances. The infant recovered without further treatment and is now 3 months old and thriving.

Comparative titrations of the alpha and beta antibodies in the sera from Cases 1 and 2 were carried out by the agglutination and conglutination techniques,¹² and the results are shown in Table I.

In the titrations carried out by the agglutination technique, it was found that the ratio of the antibody titers of the maternal and infants' sera was 8:1 in Case 1 and 64:1 in Case 2. These ratios compare favorably with those previously reported by Wiener and Silverman¹² for alpha and beta agglutinins in normal pregnancy. On the other hand, in tests carried out by the conglutination technique, the ratios of the titers approximated unity. The reason

PERMEABILITY OF THE HUMAN PLACENTA TO ISOANTIBODIES

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IT HAS been shown that Rh-negative individuals sensitized to the Rh factor may produce either or both of two sorts of antibodies, namely, Rh agglutinins or Rh-blocking antibodies.^{1, 2} The Rh agglutinins can be demonstrated by direct tests in saline media, while the Rh-blocking antibodies can be demonstrated by either the so-called blocking test performed in a saline media or directly by the conglutination reaction.^{3, 4} The Rh agglutinins behave as if they are bivalent (or multivalent) in the chemical sense, and in tests performed in saline media agglutination occurs directly, presumably because the antibody molecules link the red cells together.^{5, 6} On the other hand, the blocking antibodies behave as if they are univalent (therefore the alternative name glutinins for these antibodies) and coat the red cells, preventing the action of the Rh agglutinins in the so-called blocking test.¹ The Rh-blocking antibodies or glutinins can be demonstrated directly in tests performed in the presence of concentrated plasma or serum, because these media seem to contain a third component, conglutinin, probably a colloidal aggregate of plasma proteins analogous to the third component in hemolytic systems.³

This hypothesis leads to the concept that Rh agglutinins are larger molecules than Rh glutinins (blockers), which, if true, has important implications in the pathogenesis of erythroblastosis fetalis.⁷ One would expect that the univalent Rh antibodies (blockers) should be capable of traversing the placenta more readily than bivalent Rh antibodies (agglutinins). This hypothesis is difficult to test in cases of erythroblastosis due to Rh sensitization, because any Rh antibody passing into the fetal circulation would be immediately adsorbed by the infant's Rh-positive cells and, therefore, would not be demonstrable in the infant's serum except where excessive amounts (more than enough to coat all the erythrocytes) have been acquired from the mother. Nevertheless, we have succeeded^{3, 8} in demonstrating the presence of univalent Rh-antibody blockers (or glutinin) coating fetal and infant's red cells and also present in excess in infant's serum for periods up to a month after birth,³ indicating that these antibodies do gain access to the fetal circulation more readily than Rh agglutinins.

The idea suggested itself to test the hypothesis by comparing the alpha and beta antibody titers in maternal and newborn infant's sera. For this purpose, normal women with normal infants were not suitable, because the maternal serum in such instances would be expected to contain only natural alpha and/or beta antibodies.⁹ These sera would therefore be expected to contain

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IN MATERNAL SERUM AND NEWBORN INFANT'S SERUM

DILUTIONS OF NEWBORN INFANT'S SERUM									RATIO OF TITERS
1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	TITER	
-	-	-	-					0	-
++	+	-	-					4	8
-	-	-						0	-
+++	++	++	--	++	-+	+	-	128	1
-	-	-						0	-
-	-	-						1	64
-	-	-						0	-
+++	++	++	++	++	-+	+	-	96	1.3

pepsin, so that injections of therapeutic horse serum or diphtheria toxin-antitoxin could bring about A-B sensitization. Moreover, such antigens are present in many bacteria and animal parasites,¹⁵ so that certain infections could cause A-B sensitization. The second patient did give a history of a severe attack of pneumonia several years before her first pregnancy, and possibly this was the source of her sensitization.

It may seem somewhat puzzling that in both cases the univalent beta antibodies were more readily demonstrable in the maternal serum by the conglutination method than were the univalent alpha antibodies. The following plausible explanation for this apparent paradox suggests itself. It would be expected that univalent antibodies in general would continue to pass through the placenta from mother to fetus until their concentration in the fetal circulation became equal to that in the maternal circulation, thus setting up an equilibrium. In the case of the beta antibodies, such an equilibrium could be reached quickly; but in the case of the alpha antibodies, the antibodies would be neutralized or absorbed by group substances in the Group A fetus' body, delaying the attainment of equilibrium and at the same time gradually depleting the mother's body of the alpha antibodies.

SUMMARY

Two cases of erythroblastosis due to A and B sensitization are described. Comparative titrations by the agglutination and conglutination techniques of the alpha and beta antibodies in the maternal and infants' sera indicate that glutinins (univalent antibodies) traverse the placenta more readily than agglutinins (bivalent antibodies). This supports the hypothesis that glutinins (or blockers) are comprised of smaller molecules than agglutinins.

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TABLE I. COMPARISON OF THE ALPHA AND BETA ANTIBODIES

CASE NO.	TECHNIQUE OF TESTS	TEST CELLS	DILUTIONS OF MATERNAL SERUM									
			UNDILUTED	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	TITLE
1*	Agglutination	{ A ₂	++±	++	++	+±	+	-	-			16
		{ B	+++	+++	+++	++±	++	+	-	-		32
	Conglutination	{ A ₂	++±	++±	++	+±	+	-	-			16
		{ B	+++	+++	++	++	++	++	+±	+	-	128
2†	Agglutination	{ A ₂	Hem.	+	++±	++±	++	+±	+	-		64
		{ B	Hem.	++±	+++	++±	++	++	+	-		64
	Conglutination	{ A ₂	++±	+++	++±	++	++	++	+±	±	-	96
		{ B	Hem.	++±	++±	++	++	++	+±	+	-	128

Hem., Complete or almost complete hemolysis. Plus signs indicate degree of clumping (agglutination or conglutination). The tests were set up in small tubes in the usual manner; readings were taken after one hour's incubation in the water bath at body temperature.

*Mother, Group O; child, Group A. The maternal and the infant's blood samples in this case were both collected forty-eight hours after delivery.

†Mother, Group O; child, Group A. The maternal blood sample in this case was collected during labor; the infant's blood sample from the umbilical cord immediately after delivery.

for the discrepancy is that the two tests measure two different sorts of antibodies. The former test detects bivalent antibodies (agglutinins), while the latter test also detects univalent antibodies (glutinins). These results demonstrate, therefore, that glutinins traverse the placenta far more readily than agglutinins, in accordance with the expectations under the hypothesis that the former are comprised of smaller molecules.

It is of interest to point out that the serum of the infant of Case 2 caused hemolysis in the agglutination test despite its low titer in saline media. This may serve to explain the observation that the isohemolysin titer is not perfectly correlated with the isoagglutinin titer, because these two reactions are apparently due to two different sorts of antibodies. The tendency of isohemolysis to occur in high-titered agglutinating serum may be explained by the fact that such sera would be more likely to contain immune antibodies.

The fact that in both cases described in this paper first-born infants were erythroblastotic calls for some comment. As has been pointed out elsewhere,⁷ fetal red cells and fetal products probably gain access to the maternal circulation principally during labor and only rarely during pregnancy. Thus, maternal sensitization by pregnancy would ordinarily occur only at parturition and too late to affect the first infant. Therefore, for the first-born to be affected, some mechanism of isosensitization other than by the pregnancy itself would have to be postulated. In the first case, the patient stated that two years previously she had had an ovarian tumor removed and was given several post-operative transfusions of commercial, pooled human plasma. Since it has been found that such transfusions stimulate a rise in the alpha and beta isoagglutinin titers, this is a satisfactory explanation for the existence of sensitization in the first case. With regard to the second case, the source of the sensitization could not be traced as definitely. However, it should be pointed out that A-like and B-like antigens are ubiquitous in nature, so that there would be many opportunities for A-B sensitization to occur in the course of a lifetime.¹⁴ For example, such antigens are present in horse serum and pig

THE QUANTITATIVE DETERMINATION OF A PEPSIN-LIKE SUBSTANCE IN THE URINE OF NORMAL INDIVIDUALS AND OF PATIENTS WITH PERNICIOUS ANEMIA

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THE first report of a proteolytic substance in urine was made in 1861 by Brücke,¹ who noted that fibrin was digested when incubated with urine. Similar experiments were performed by Grützer² in 1887, Gehrig³ in 1886, and Hoffmann⁴ in 1887. The character and origin of this ferment were studied further by Leo⁵ in 1885, Schnapauf in 1888,⁶ and Loeper and Esmonet⁷ in 1908. On the basis of this work, there developed two theories: first, that the enzyme was pepsin and was derived from the intestine; second, that the active principle was a proferment probably secreted directly into the blood stream by the gastric mucosa. Frouin,⁸ in 1904, working with a gastrectomized dog and another in which the stomach had been isolated with the blood supply intact, concluded that urinary pepsin is of gastric origin, that it enters the blood stream at the level of the stomach, and that no resorption occurs in the intestine. After this simple and critical experiment, the progress and validity of investigation in this field were hampered by analytic methods not sufficiently accurate to insure other than qualitative results, and attempts to relate the urinary ferment quantitatively with gastric secretion of pepsin, either in health or in disease, proved confusing.

It was not until 1924 that Gottlieb⁹ devised an accurate method for the simultaneous study of pepsin in the stomach, in the blood, and in the urine. His method is not fully described, but it consisted essentially of digestion of edestin with comparison to the activity of a known solution of Armour's pepsin. Applying this method to a small group, Gottlieb reported that high gastric acidity was associated with a significantly higher urinary pepsin than was low gastric acidity. He also noted a correlation between gastric and urinary pepsin. Gottlieb further reported that alkalinization of the urine to a pH of 8 or 9 did not inactivate the enzyme and concluded that the substance was pepsinogen. Three years later Teschendorf¹⁰ studied the pepsin content of the gastric juice and urine in patients with achylia. Here again, the number of cases was small and the results conflicting.

The purpose of the present paper was to study further the excretion of urinary pepsin in normal individuals and in patients with achylia (pernicious anemia) with the use of improved methods. We found that the modification of the Anson and Minsky method (Beazell and associates¹¹) could be satisfactorily adapted for the determination of urinary pepsin. This method was employed by Bucher¹² in a preliminary report of a study on young women. Our results show

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Zerfas¹³ in studies of gastric enzymes. A larger series must be studied before such a relationship can be excluded.

The effect of specific gravity on the urinary peptic activity was negligible. Twelve normal urine specimens with the highest tyrosine values had an average specific gravity of 1.019 as compared to 1.016 in the twelve normal urines with the lowest tyrosine values. The acidity of the urine was likewise unrelated to the peptic activity. Many specimens with a pH of 7.0 or over had normal tyrosine values. This is in agreement with the idea that the enzyme in urine is a proferment or pepsinogen.

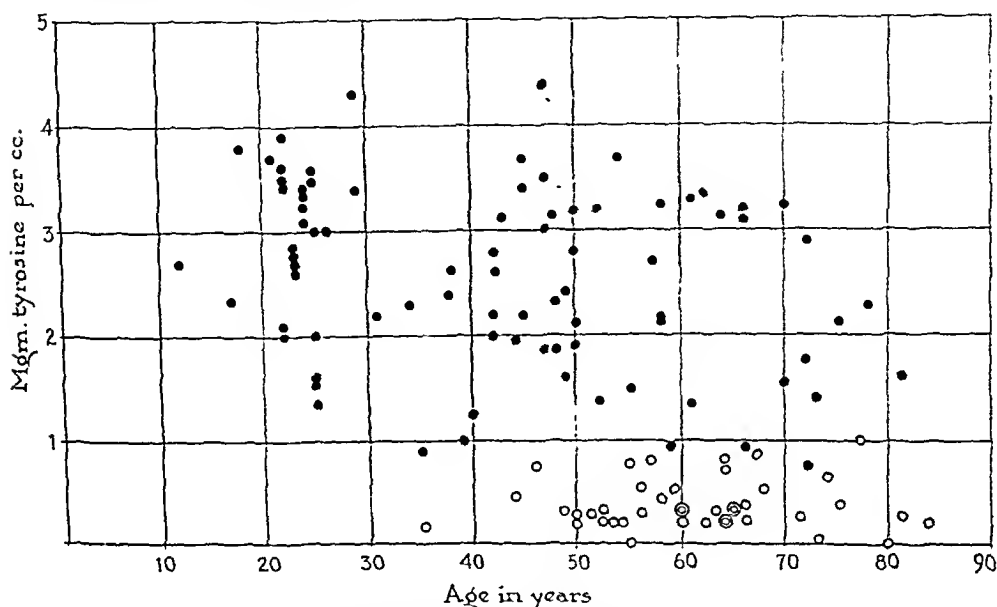


Fig. 1.—Relation of tyrosine value to age. Dots indicate normal individuals; open circles, patients with pernicious anemia.

COMMENT

The term pepsin-like substance has been used in the title because of the possibility that the enzymatic action of the urine was not due to pepsin. However, the facts that (1) the digestion of hemoglobin solution occurs at a low pH, (2) the enzyme concentration of urine of patients with achylia gastrica is low as compared to normal individuals, and (3) the substance is not irreversibly inactivated in a neutral solution strongly suggest that the enzyme is related to pepsin and occurs normally as pepsinogen. Further evidence for this conception is presented in the older literature.^{5, 9}

Although determinations of the gastric pepsin were not made in our subjects, it might be inferred that the quantitative urinary pepsinogen roughly parallels the peptic activity of the gastric juice. Patients with pernicious anemia, who are known to have a marked diminution of gastric pepsin,¹² had a correspondingly low urinary pepsin. Patients with achlorhydria following histamine stimulation but not having pernicious anemia are known to have some decrease in the gastric pepsin but not to the extent observed in pernicious

a striking decrease in the pepsin or pepsin-like substance in the urine of patients with pernicious anemia as compared to normal individuals.

METHODS AND MATERIAL

All determinations were made on fasting morning urine specimens. The method of determining pepsin activity involves the incubation at 37° C. of 5 c.c. of a known reproducible hemoglobin substrate, buffered at a pH of 1.7, with 1 c.c. of urine for sixteen hours. To prevent bacterial growth, the mixture is overlaid with toluene. After incubation the solution is treated with 10 per cent trichloroacetic acid to precipitate the undigested hemoglobin. The phenolic digestion products in the filtrate, presumably chiefly tyrosine, are then quantitatively determined by the photoelectric colorimeter and compared with a known solution of tyrosine. Because phenolic compounds are normally found in the urine, a blank consisting of 1 c.c. of urine, similarly treated but previously inactivated by heating for five minutes in a boiling water bath, is concomitantly assayed, and the results are subtracted from the reading of the active sample. The peptic activity is expressed as milligrams of tyrosine produced by 1 c.c. of urine. The tyrosine equivalent has been carefully investigated throughout the range of concentrations with which we are dealing and has been found to bear nearly a straight line relationship.

The normal series consisted of eighty-two individuals comprising both sexes and all age groups from the second to the eighth decades. The younger subjects were for the most part medical students and clinic personnel, while the older group were ambulatory and hospital patients who were in comparatively good health and had no symptoms referable to the gastrointestinal tract. Gastric analyses were not performed in these persons.

The pernicious anemia series consisted of forty-one patients of both sexes from the hematology clinic. All of these patients were receiving parenteral liver therapy and were in complete remission. Originally they had had the typical blood picture of pernicious anemia, had shown complete achlorhydria following histamine stimulation, and had responded specifically to liver therapy.

RESULTS

Individual values for the peptic activity of urine are plotted against the age of the subjects in the spot curve (Fig. 1). The normal series have a wide range of values which is compatible with the expected variation in gastric function seen in an unselected group. The values ranged from 0.7 to 4.4 mg. with an average of 2.4 mg. tyrosine per 1 c.c. urine for sixteen hours. Among the patients with pernicious anemia, the values ranged from 0 to 1.0 mg. with an average of 0.5 mg. Taking 1.0 mg. as the maximum value in pernicious anemia, only five of the eighty-two normal subjects had a peptic activity at or below this level. Gastric analysis might possibly have shown diminished gastric function in these five patients.

A decrease in urinary pepsin might have been anticipated in the advanced age group; a clear-cut correlation, however, could not be made (Fig. 1), and plotting by decades gave results similar to those obtained by Helmer, Fouts, and

HEMOCHROMATOSIS

REVIEW OF LITERATURE AND PRESENTATION OF A CASE WITHOUT PIGMENTATION OR DIABETES

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HEMOCHROMATOSIS is a rare disorder of iron metabolism occurring predominantly in middle-aged men, characterized by a clinical triad of hepatic enlargement, diabetes, and pigmentation of the skin. This case, occurring in a 14-year-old white boy without pigmentation of the skin and diabetes, is being presented because of its atypical clinical features.

CASE REPORT

A 14-year-old white boy was admitted for the first time to the University Hospital on Feb. 1, 1944, with the chief complaint of upper respiratory infection. For the past six years he had been anemic and had been treated with liver and iron. The anemia was of the hypochromic type and gradually progressed. For six months before the first admission he had complained of progressive weakness and fatigue. A few days before this admission he developed an upper respiratory infection with postnasal drip, hacking cough, and frontal headache. On physical examination the spleen was palpated three fingerbreadths below the right costal margin and was slightly tender. The blood count showed: red blood cells, 2.5 million per cubic millimeter; hemoglobin, 3.9 Gm. per 100 c.c.; reticulocytes, 1.2 per cent; and white blood cells, 4,000 per cubic millimeter with 64 per cent neutrophils, 27 per cent lymphocytes and 9 per cent monocytes. There were 350,000 platelets per cubic millimeter. The fragility of the red blood cells was normal. A sternal puncture revealed hyperplasia of the erythroid elements. He was Rh negative. The results of an adrenalin test were interpreted as showing excessive sequestration of red blood cells, white blood cells, and platelets. On the eighteenth hospital day a splenectomy for Banti's disease was performed. At the time of the operation the liver was enlarged but did not appear to be cirrhotic. Microscopic sections of the spleen showed reticulum-cell hyperplasia with fibrosis and dilatation of the sinusoids; hemosiderin was present in increased amounts. Postoperatively the red blood cells were stabilized at 4.5 million per cubic millimeter and the white blood cells at 7,000 per cubic millimeter. After an uneventful course the patient was discharged on the fourteenth postoperative day.

The second admission occurred three months after the first. At this time the patient complained of weakness with anorexia and nausea. He was pale, weak, and listless. The liver was not palpable. The admission blood count showed: red blood cells, 1.75 million per cubic millimeter; hemoglobin, 3.8 Gm. per 100 c.c.; reticulocytes, 1.6 per cent; and white blood cells, 15,000 per cubic millimeter with 60 per cent neutrophils and 40 per cent lymphocytes. The patient was given four transfusions. The red blood count rose to a maximum of 2 million per cubic millimeter and the hemoglobin to 8 Gm. per 100 c.c. He was discharged on the seventh hospital day.

The third admission was two months later. The patient was pale, poorly nourished, and somewhat icteric. The liver was palpable two fingers below the right costal margin. On three successive days he received 1,000 c.c. of suspended red blood cells. No blood counts were recorded for this admission. The patient was discharged greatly improved.

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anemia.¹⁴ We studied the urinary pepsin in fourteen women patients with true achlorhydria, all of whom had recovered from an iron deficiency anemia. The peptic activity expressed as tyrosine ranged from 0.3 to 3.2 mg. with an average of 1.2 mg. (average for normal subjects, 2.4 mg.). Eight of the fourteen patients had values above 1 mg., the upper limit seen in pernicious anemia. Further evidence thus is offered for a quantitative relation between gastric and urinary pepsin.

The extremely low values for peptic activity of the urine that were observed in patients with pernicious anemia might make the test applicable for clinical purposes. The method is simple and is performed on a single fasting urine specimen. In the forty-one patients with pernicious anemia, the peptic activity of 1 c.c. urine expressed as milligrams of tyrosine showed values of 1 mg. or less, whereas only 6 per cent of eighty-two normal persons⁶ fell within this range. Therefore, in a patient suspected of having pernicious anemia, a tyrosine level above 1 mg. would be good evidence against this diagnosis.

SUMMARY

1. Quantitative estimations of a pepsin-like substance in the urine were made by a modification of the Anson and Mirsky method.
2. The peptic activity of the urine was markedly diminished in patients with pernicious anemia as compared to normal subjects.
3. The possible diagnostic application of the test is discussed.

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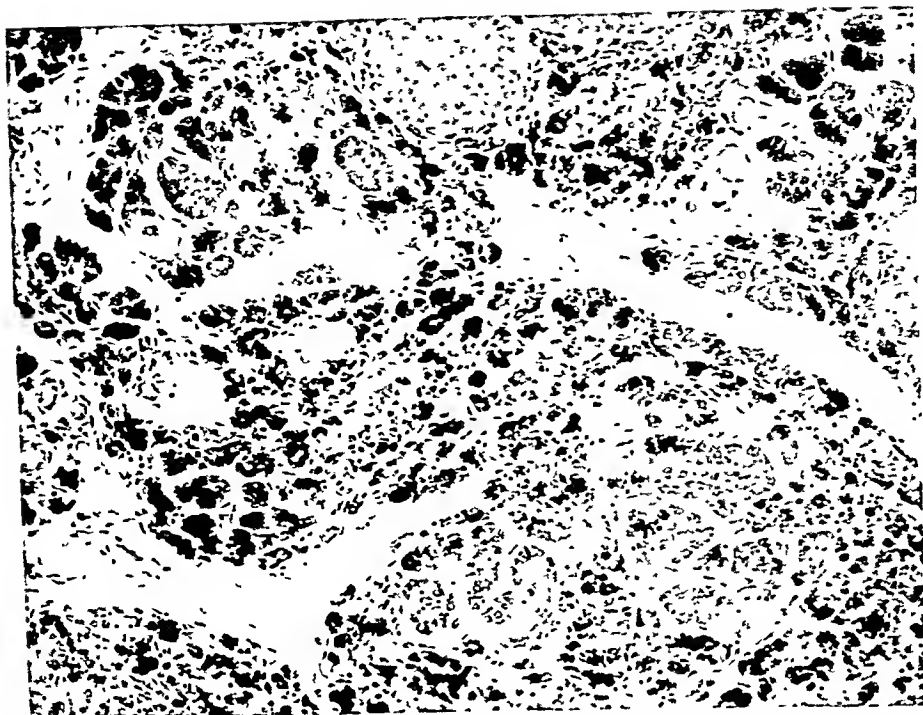


Fig. 1.—Pancreas. Section shows diffuse hemosidero-fibrosis with practically no involvement of islets. (Iron stain.)

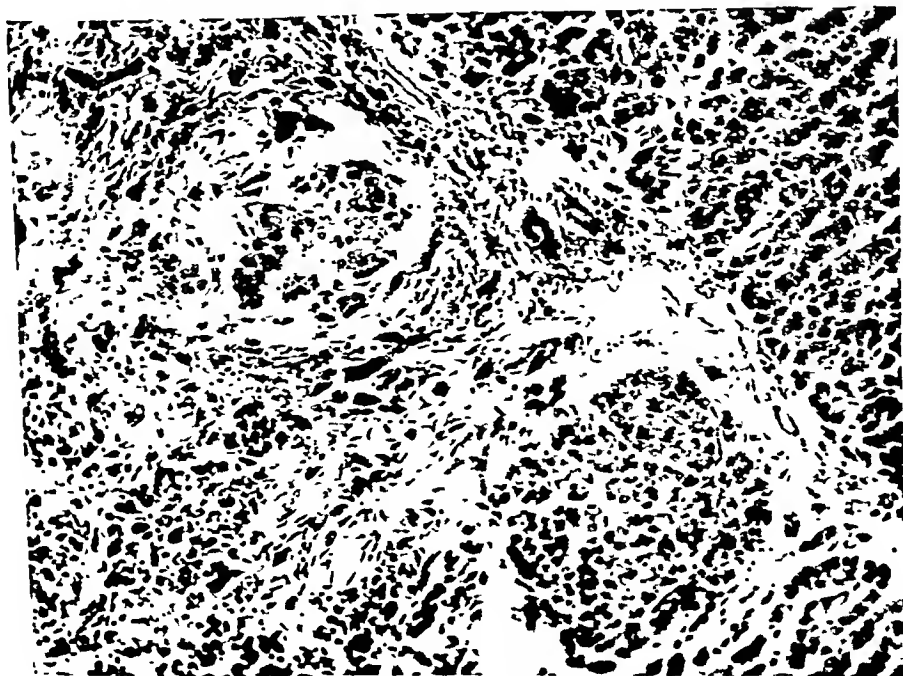


Fig. 2.—Liver. Section shows cirrhosis and hemosiderosis. The pigment is deposited in greater quantities in large mononuclears in the portal canals. It is also present in large amounts in the parenchymal cells and Kupffer cells. (Iron stain.)

a hemorrhagic infarction of the lower two-thirds of the small bowel with lesser involvement of the upper portion. The loops were heavy and filled with a bloody material. The wall was hemorrhagic. The mesenteric fat was edematous and thick, measuring up to 3 to 4 cm. in thickness. Examination of the main superior mesenteric artery and its branches failed to reveal any evidence of thrombus. The fairly soft and yellow-gray thrombus in the superior mesenteric vein was previously described. The adrenals showed lipoid depletion. Each kidney weighed about 190 Gm. They were pale and soft. The bladder, ureters, and sex organs were normal. The aorta showed no evidence of atherosclerosis or syphilis. The bone marrow was red. The head and spinal cord were not examined.

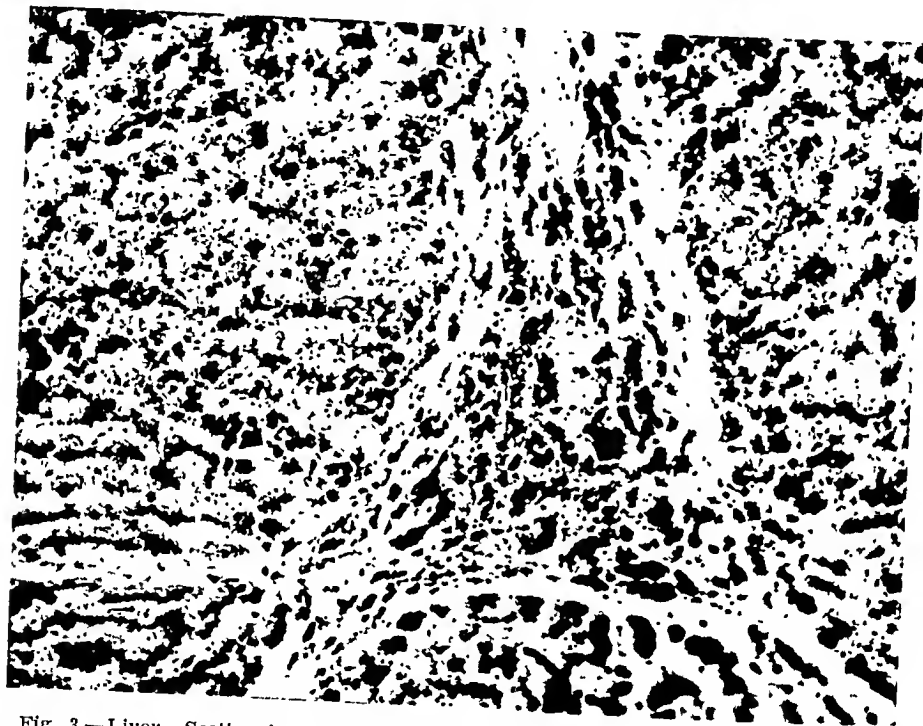


Fig. 3.—Liver. Section is similar to that illustrated in Fig. 2 and shows cirrhosis and pigmentation. Large quantities of iron pigment are readily seen with routine stains such as this. (Hematoxylin and eosin.)

Microscopic Findings.—The heart showed parenchymatous degeneration. The lungs showed thickening and congestion of the alveolar septa and a few small arterial thrombi. The spleen showed congestion, reticulum-cell hyperplasia, and hemosiderosis. No lymphoid tissue was noted in the splenules. The liver showed a portal type of cirrhosis with excessive iron pigmentation in the periportal areas, Kupffer's cells, and parenchyma. The greatest amount of iron was in the portal canals where the iron was found mainly in phagocytic macrophages. There was a recent organizing thrombus of the portal vein with periportal chronic inflammation. The pancreas showed patchy degeneration with hemosidero-fibrosis. The islets were uninvolved and were negative for pigment with iron stains. Other changes were fatty infiltration and endarteritis of the splenic artery. The gastrointestinal tract sections showed congestion, edema, and early infarction of the small bowel. The kidneys showed parenchymatous degeneration. Sections of the sex organs revealed no significant histopathologic changes. The bone marrow showed an increase in megakaryocytes. Special stains for iron were made of all organs. Small amounts of iron pigment were also found in epithelial cells of prostate, heart, and in the epithelial cells of the collecting tubules of the cortex. Moderate amounts of iron were found in the outer layers of the cortex of the adrenal. Fairly large

amounts of iron were found in the splenule. Quantitative analysis of the liver showed 2 per cent by weight, or 47 Gm. of iron in the liver. No sections of the skin were submitted for histologic examination.

Final Diagnosis.—Hemochromatosis with hemosidero-fibrosis of the liver and pancreas and generalized hemosiderous; portal cirrhosis of liver; recent thrombosis of portal and superior mesenteric veins with hemorrhagic infarction of the lower three-fourths of the small intestine; atelectasis of the right lower lobe; Ghon tubercle in the lower lobe of right lung; absence of spleen (surgical removal).

DISCUSSION

Diagnosis.—From the point of view of differential diagnosis this case represents several interesting problems. The age of the patient is much younger than the usual recorded age of 45 to 50 years. In the series of Sheldon¹ the earliest recorded age for the appearance of the fully developed disease is 20, although symptoms of the disease did appear at 7, 16, 17, 18, and 19 years of age. Sheldon further states that the occasional early emergency of symptoms may be expected.

The pathologic interpretation of the case is that of hemochromatosis. The diagnosis is based upon the findings of pigment cirrhosis of the liver, fibrosis and pigmentation of the pancreas, and siderosis of other organs.

Two of the three classic signs and symptoms, diabetes and pigmentation of the skin, were absent. In Sheldon's series pigmentation of the skin constituted the first symptom of disease in 25.7 per cent of the cases and was found in 83.8 per cent of his series. It was the first symptom noticed in 40 per cent of the series published by Butt and Wilder.² Lisa and Hart,³ on the other hand, emphasized that a certain number of cases may not show any skin pigmentation and that as many as 75 per cent of early cases will have no evidence of bronzing. Diabetes was found in 25.7 per cent of the patients in Sheldon's series as the initial symptom and was finally present in 70 per cent of his cases. Eighty-six per cent of the Mayo series² gave laboratory evidence of diabetes. According to Karsner⁴ one out of every 2,000 to 3,000 persons with diabetes show hemochromatosis. Enlargement of the spleen, which in our case was one of the first noted organic changes, is known to occur in hemochromatosis. In Sheldon's series the spleen was enlarged in 60 per cent of the cases, while an enlargement of the liver was diagnosed in 92 per cent of the cases.

A particularly interesting finding in our case was the presence of anemia which preceded the onset of all other symptoms by approximately six years. From the laboratory data we can conclude that it was a hypochromic microcytic anemia with no evidence of marrow hypoplasia. The values for the red blood cells and hemoglobin alone were depressed. The reticulocytes were either normal or moderately elevated. The platelets were normal before splenectomy and greatly elevated after splenectomy. The white blood cells were decreased in number before splenectomy and fluctuated considerably afterward. These findings would support a diagnosis of an iron deficiency anemia as against an aplastic type of anemia. Only six cases of Sheldon's series showed a red blood count below 2.5 million per cubic millimeter. Anemia associated with hemochromatosis has been observed by other authors, and the combination of both

conditions has been considered more than accidental by Zeltmacher and Bevans.⁵ However, their case, as well as those of Kark,⁶ Sturgis,⁷ and Mackey,⁸ and the three cases of Bomford and Rhoads⁹ were aplastic in type.

The cause of death in our case must be considered quite unusual and may be one of the late complications of splenectomy in portal obstruction. The thrombosis was not accompanied by a pyogenic process in the portal system. It is interesting that we had the opportunity at operation to study the spleen at a stage where no evidence of pigment cirrhosis was noted. Microscopically the spleen showed an increased amount of iron pigment together with changes typically associated with the spleen in liver cirrhosis.

Pathogenesis.—Sheldon, who made an exhaustive study of the disease, believes that hemochromatosis is a congenital disease and consists chiefly of a fundamental disorder in the iron metabolism with accumulations of small amounts of pigment over a long period of time. Consequently, it takes 40 to 50 years for the emergence of symptoms, although occasionally symptoms occur at an earlier date. In his series of 311 cases there was no evidence of increased hemolysis, such as increased icterus index, reticulocytosis, or increased bilirubin excretion in the urine, nor was there any evidence of the action of a toxin. The majority of writers on this subject concur with this opinion. Rosenthal¹⁰ believes that the fundamental disturbance is the inability of the liver to change ferrie to ferrous iron. Mallory's¹¹ work on experimental animals led him to believe that intoxication with copper with resultant destruction of erythrocytes and liberation of iron from hemoglobin is an important factor in the production of hemochromatosis. Smith and Gault¹² suggest that hemochromatosis might be analogous to von Gierke's disease, the iron entering into the cell normally and being transformed into a form incapable of excretion.

An interesting theory has been suggested by a group of authors⁵ who described hemochromatosis in association with aplastic anemia. They believe that in those cases observed after they have been kept alive by a large number of blood transfusions increased hemolysis may be the etiologic factor of hemochromatosis, while the cause of anemia is probably obscure. It is suggested that the iron derived from the destruction of intrinsic and transfused blood is deposited in a cirrhotic liver, thus producing a picture indistinguishable from hemochromatosis. As the pancreas becomes involved in this process, diabetes might develop.

Whether hemochromatosis can be produced by an increased amount of blood transfusions in various anemic conditions has not been definitely answered. Sturgis expresses the opinion that in his case hemochromatosis did develop after 137 blood transfusions. Mackey and Kark likewise believe that the hemochromatosis in their cases is directly related to the amount of iron administered in transfusions. Of the other cases of aplastic anemia developing hemochromatosis, Bomford and Rhoads make no direct statement as to the relationship of hemochromatosis to multiple transfusions, while Zeltmacher and Bevans are unable to account for the total amount of iron found at autopsy on the basis

of transfusions alone. Kilduffe and DeBailey¹³ state that occasionally patients who receive transfusions over prolonged periods of time develop hemochromatosis, but they do not give details. However, others experienced in the consequences of blood transfusion would not generally accept such etiologic relationship.¹⁴⁻¹⁷ Experimental attempts to reproduce hemochromatosis by repeated transfusions, by hemolytic agents, and by injection hemoglobin or dialized iron have not been successful.¹⁸⁻²⁰

In our case the patient received a total of 6 liters of blood over a nine months' period, or 2.75 Gm. of iron. The amount of iron recovered from the liver was 47 Gm. From these calculations it is clear that the pigmentation cannot be explained by the retention of transfused iron.

A recent article by Gillman and Gillman²¹ from South Africa, on the structure of the liver in pellagra, is most interesting and if confirmed would constitute a new approach to the study of hemochromatosis. On the basis of 400 liver biopsies in 120 patients with pellagra, they conclude that hemochromatosis is one of the commoner sequelae of pellagra (15 per cent of the cases). The hemosiderin and hemofuscin pigments are formed in association with changes in the mitochondria, ultimately leading to the disruption of the mitochondria. This profound disturbance of intracellular metabolism is related to some forms of malnutrition. Hemochromatosis is therefore considered as an acquired rather than as a congenital disease. Further observations, based on a series of 700 livers from autopsies in sudden and violent deaths (unreported), lead them to believe that hemochromatosis is a common disease in Africans. They attribute this to the totally inadequate diets of these natives.

There is inadequate information to discuss our own case from a nutritional point of view in line with Gillman and Gillman's recent concept. Notes on the first two admissions described the patient as being well nourished and on the last two admissions as being poorly nourished. The autopsy protocol describes the patient as being fairly well nourished.

SUMMARY AND CONCLUSION

1. An atypical case of hemochromatosis without pigmentation of the skin and diabetes as occurring in a 14-year-old white boy is presented.

2. The diagnosis is based upon the findings of pigment cirrhosis of the liver and pancreas and siderosis in other organs.

3. A particularly interesting feature in our case is the presence of a severe anemia which preceded the onset of other symptoms by approximately six years. From the laboratory data we classify this as a hypochromic microcytic anemia of the iron deficiency type.

4. In the more recent literature the association of hemochromatosis with cases of aplastic anemia has been noted. The possible relationship between hemochromatosis and the multiple transfusions given to these cases has been suggested. At the present time we do not believe that this question has been definitely answered.

5. No such relationship is present in our case. A total of 2.75 Gm. of iron were contained in the transfused blood. The liver at autopsy contained 47 Gm.

6. In a recent paper Gillman and Gillman propose a nutritional basis for the pathogenesis of hemochromatosis.

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LABORATORY METHODS

A SIMPLE METHOD FOR PERFORMING A WASSERMANN TEST ON ANTICOMPLEMENTARY SERUM

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IN ALL clinical laboratories serologists have obtained anticomplementary reactions in the performance of the Wassermann tests. The common practice in such cases is to report the test as anticomplementary and to request that another specimen of blood be taken. Usually, the next specimen is found to be satisfactory. There are also some cases in which anticomplementary reactions are obtained on several successive specimens of serum.

An attempt was made to study these tests and to devise a simple method for performing a Wassermann test on anticomplementary serum, in order to be able to report the test as negative, doubtful or positive and to eliminate the necessity of taking further specimens of blood from the patient.

Since the criterion for the anticomplementary reaction is found in the control tube, where no antigen is added, it becomes apparent that the patient's serum has the ability to "fix" complement by itself. It was found expedient, therefore, to saturate the patient's serum with complement by incubation with undiluted complement at 37° C. and then to destroy the excess complement by inactivating for thirty minutes at 56° C. At this point the serum is ready for the Wassermann test. The ability of the serum to "fix" complement has now been thoroughly satisfied, and further fixation in the control tube (without antigen) is no longer possible. The usual amount of complement is added to all of the tubes in the performance of the Wassermann test on the complement-saturated serum.

This conception has been amply borne out experimentally. The tests were performed on 200 anticomplementary serums over a period of about fifteen months. Included with each setup of anticomplementary bloods were known negative and positive serums as controls for the method employed. Fresh specimens of blood were also obtained from those patients having anticomplementary reactions in order that routine Wassermann and Kahn tests could be performed as additional controls for the complement-saturation method.

METHOD

To 2 c.c. of anticomplementary serum add 0.3 c.c. of undiluted complement and place in 37° C. water bath for thirty minutes. Remove and place in 56° C. water bath for thirty minutes to destroy excess complement. Perform a Wassermann test on the serum in the usual manner. The Kolmer three-tube test was employed in this laboratory.

From the New Rochelle Hospital Laboratory; James Denton, M.D., Director.
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It will be found that in the great majority of cases this proportion of serum and undiluted complement will suffice to saturate the serum. However, if an anticomplementary reaction is again obtained, the procedure can be repeated with an additional dose of 0.1 to 0.2 c.c. of undiluted complement.

RESULTS

All bloods that were anticomplementary we were now able to report as negative, doubtful, or positive. In each case the control tube showed complete hemolysis.

The addition of undiluted complement to known negative and positive serum had no deleterious effects. The results on these serums were the same with and without complement saturation.

TABLE I. REPRESENTATIVE SAMPLE OF RESULTS OF COMPLEMENT-SATURATION TESTS ON ANTICOMPLEMENTARY SERUM AND CONTROL WASSERMANN TESTS ON FRESH SERUM AND KAHN TESTS

CASE	WASSERMANN ON ANTICOMPLEMENTARY SERUM	WASSERMANN ON FRESH SPECIMEN OF SERUM	KAHN
1	Positive	Positive	Positive
2	Positive	Positive	Positive
3	Positive	Positive	Positive
4	Positive	Positive	Positive
5	Positive	Positive	Positive
6	Positive	Positive	Positive
7	Negative	Negative	Doubtful
8	Doubtful	Doubtful	Positive
9	Positive	Positive	Positive
10	Positive	Positive	Positive
11	Positive	Positive	Positive
12	Positive	Positive	Positive
13	Positive	Positive	Positive
14	Negative	Negative	Negative
15	Positive	Positive	Positive
16	Positive	Positive	Positive
17	Positive	Positive	Positive
18	Doubtful	Doubtful	Doubtful
19	Positive	Positive	Positive
20	Positive	Positive	Positive
21	Positive	Positive	Positive
22	Positive	Positive	Positive
23	Positive	Positive	Positive
24	Negative	Negative	Negative
25	Positive	Positive	Positive

The Wassermann tests on the anticomplementary serums checked very closely with the Kahn tests, as well as with repeated Wassermann tests performed on fresh serum. In Table I is given a representative sample of the results obtained in this study. It is noteworthy that the majority of serums that were anticomplementary were positive with the method described.

Although the method could be applied to anticomplementary serums that are very badly hemolyzed or greatly contaminated with bacteria, it is not advisable to run these. In such cases it is best to obtain fresh specimens of blood, since this is encountered very infrequently.

SUMMARY AND CONCLUSION

1. A simple procedure has been given which permits the performance of a Wassermann test on anticomplementary serum and which eliminates the necessity of taking further specimens of blood from the patient.

2. The procedure is based on the saturation of the serum with undiluted complement to satisfy the "fixing" properties of the serum. The excess complement is then destroyed, and a Wassermann test is performed in the usual manner.

3. In all cases complete hemolysis was present in the control tube, and the tests were reported as negative, doubtful, and positive.

4. Known negative and positive serums were treated in the same manner as the anticomplementary serums in order to check on the effects of complement saturation. The results of these tests were the same with and without complement saturation.

5. There was excellent correlation between the Wassermann test on anticomplementary serum and the repeated Wassermann test on fresh serum and the Kahn test.

A MECHANICAL DEVICE TO FACILITATE ELECTROCARDIOGRAPHIC READING

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THERE are several mechanical devices in use to facilitate electrocardiographic interpretation. They are based on the idea of applying an indicated scale against the electrocardiogram, merely trying to eliminate time multiplication or counting of millimeters. The need was felt for a new instrument, simple in design, to aid in every possible phase of electrocardiographic reading without the additional use of compasses.

This instrument (Fig. 1) combines the principle of the slide rule and the caliper. It is made of plastic material and is equipped with a sliding tongue, which makes it possible to gauge distances and to compare set distances. It is also equipped with a glass lens of such focal length and size as to collect the light rays from a near-by source of light and thereby to brighten up and magnify the observed area four times, without distorting the millimeter and time lines of the electrocardiogram.

The sliding tongue and the fixed zero indicator have a longitudinal line which can be used as a base line.

The instrument has three scales which read from the right to the left. Scale 1 indicates millimeters; Scale 2 indicates the calculated time intervals; and Scale 3 indicates the calculated heart rates of a regular rhythm.

The reading is taken against the hairline on the cover glass which is mounted in a fixed aluminum frame. The gauging and comparing of distances are obtained directly on the electrocardiogram, between the edge of the wedge-shaped fixed zero-piece (A) and the edge of the wedge-shaped end of the sliding tongue (B). The scales are impressed upon the top surface of the sliding tongue, directly below the hairline. This eliminates faulty reading due to parallax, both in gauging and reading.

The instrument is applied as follows:

1. *Determination of Regularity.*—The reader is placed on the electrocardiogram with the longitudinal line over the base line. The edge of the fixed zero-piece is set over the peak of the R or S wave, and the edge of the sliding tongue is pulled out to the peak of the next R or S wave. In order to determine the regularity or irregularity of the heart rate, this interval is compared with corresponding intervals in other complexes by moving the set reader to various complexes.

2. *Reading of the Heart Rate.*—The heart rate is obtained directly by taking the reading from the heart rate scale of the R-R or S-S interval against the hairline.

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3. *Reading of Time Intervals.*—The reader remains in the same position. The time scale indicates the distances in seconds. The tongue is pulled out to the end of the wave whose time is to be measured, while the fixed zero edge coincides with its beginning. The exact time interval is read against the hairline.

4. *Reading of Millimeter Distances.*—The instrument is placed on the electrocardiogram with the longitudinal line in the direction of the millimeter lines. The reading of the height of the different waves is obtained by moving the sliding tongue from the base line to the apex of the wave and by reading the indicated millimeters against the hairline. Such set intervals can be compared with different complexes simply by moving the reader over to the various complexes.

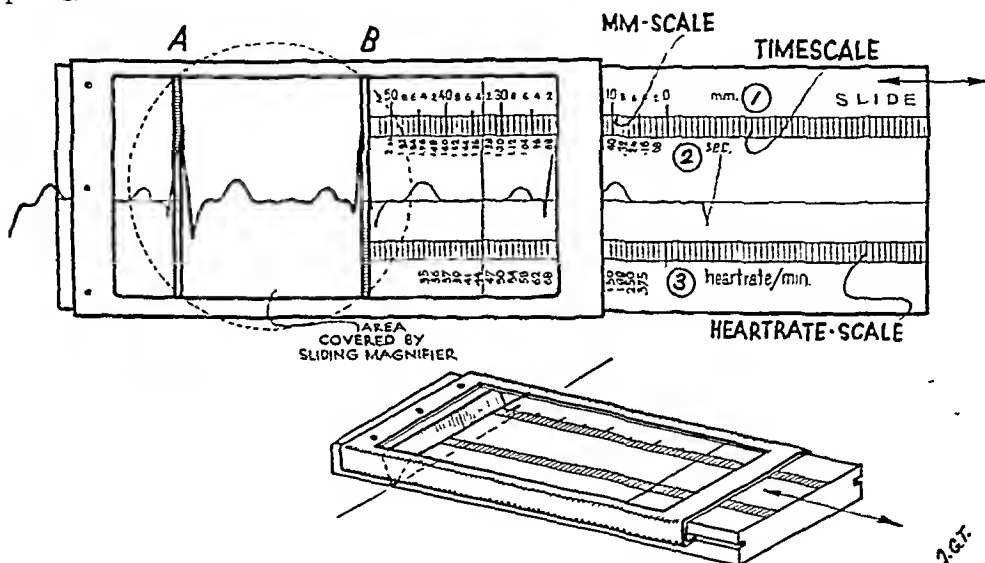


Fig. 1.*

The reader is transparent over its entire area in order to maintain visibility of the full length of the electrocardiogram under scrutiny.

This reader helps to obtain accurate measurements. It aids in compiling numerous data for electrocardiographic research and for diagnosis.

*The instrument will be manufactured by the United Optical Mfg. Corp., New York, N. Y.

AN ELECTROCARDIOGRAPHIC CALIPER CALCULATOR

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RULES for the direct measurements of heart rate (and cycle length) on the electrocardiogram are based on a fixed reference distance, implying a single and uniform camera speed. This precludes their use for cameras with different speeds and leads to errors in reading when the speed of the camera is not uniform.

The present caliper was developed to take into account such variations in camera speed and to make the instrument adaptable for a wide range of speeds. The caliper is constructed so that rates as slow as 30 can be measured readily with camera speeds of 19 to 30 mm. per second.

The caliper is illustrated in the closed position in Fig. 1 and ready for use in Fig. 2. The instrument consists of an adjustable caliper (*B*) and a double rule (*A*), the lower one giving cycle length in hundreds of a second and the upper one heart rate in beats per minute. The scale is 25 cm. long and permits the reading of heart rates between 30 and 400 beats per minute or of cycle lengths between 2.0 and 0.15 seconds. To construct the upper scale, heart rates between 30 and 400 were converted into distance, to be read from the zero point, ranging from $\frac{750}{30}$ cm. = 25 cm. to $\frac{750}{400}$ cm. = 1.875 cm. In the lower scale the subdivisions are 1.25 mm. apart. Each division represents 0.01 seconds. The total length of the scale, 25 cm., therefore contains 200 subdivisions or 2 seconds. The two sections of the rule are separated by a slot (*C*) in which the peg (*E*) can slide.

The caliper is an ordinary enlarging caliper whose longer limbs can be varied in length by the setting of the upper rods (*H* and *H'*, respectively). These rods can be adjusted by loosening the screws (*I*), and once set they are fixed in place by retightening the screws. The shorter limbs (*F*) of the caliper are 2½ cm. long, measured from the fulcrum. The longer limbs (*G*) can be varied from 16 to 25 cm. The rods of the longer limbs are constructed so as to enable them to slide easily in grooves in the fixed part of the longer limbs. One of the rods is fastened to the rule through a pivot that corresponds exactly to the zero point of the scales (*O*). The other rod, as mentioned before, can slide in the slot between the two scales of the rule.

In operating this caliper, the small limbs are applied to the electrocardiographic record so that the points are separated by time lines marking 0.60 of a second. Then the screws (*I*) are loosened and the length of the long limbs

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adjusted so that they bound the distance of 0.60 seconds in the lower scale.* The extra scale on these limbs (*S*) permits setting of both limbs to precisely the same length. The screws (*I*) are then fastened in position, and the caliper is ready for use. The small limbs are applied to mark off the desired distance (R-R or P-P intervals, etc.). The long limbs immediately will give the cycle length and heart rate. When several cycles are measured, the cycle length



Fig. 1.

can be obtained by dividing the lower scale by the number of cycles measured, and the rate can be obtained by multiplying the reading on the upper scale by the number of cycles measured. In the case of flutter and fibrillation of the auricles, a similar procedure can be used to get the auricular rate.

When used in the manner described, the caliper obviates the inconvenience of fixed rules when camera speed varies. In its construction and use in the

*The rule has an indentation (*D*) in the upper scale set it a cycle length on the lower scale of 0.60 seconds in order to facilitate this operation. A slight upward movement of the rule will remove the peg (*E*) from the notch (*D*) and permit it to slide freely in the slot of the rule.

manner outlined there is, with the usual camera speed, approximately a seven to one enlargement of the scale distance of the record. The enlargement is given by the ratio of the large to small limbs, and it varies from $\frac{16}{2.5}$ to $\frac{25}{2.5}$ or from 6.04 to 10. With a little experience this caliper calculator can be employed quickly. It is especially useful in those institutions which handle a large number of records daily, since the camera speed in a day's run of one machine is sufficiently constant so that once the caliper is set it can be used without readjustment for all of the records of that machine that day. It is also time saving in calculating average rates and cycle lengths in the presence of arrhythmias. However, it must be stressed that its proper use demands a rigorous compliance with the simple rules previously outlined. The caliper has been tested on a large series of records in the Heart Station and found satisfactory in our hands.

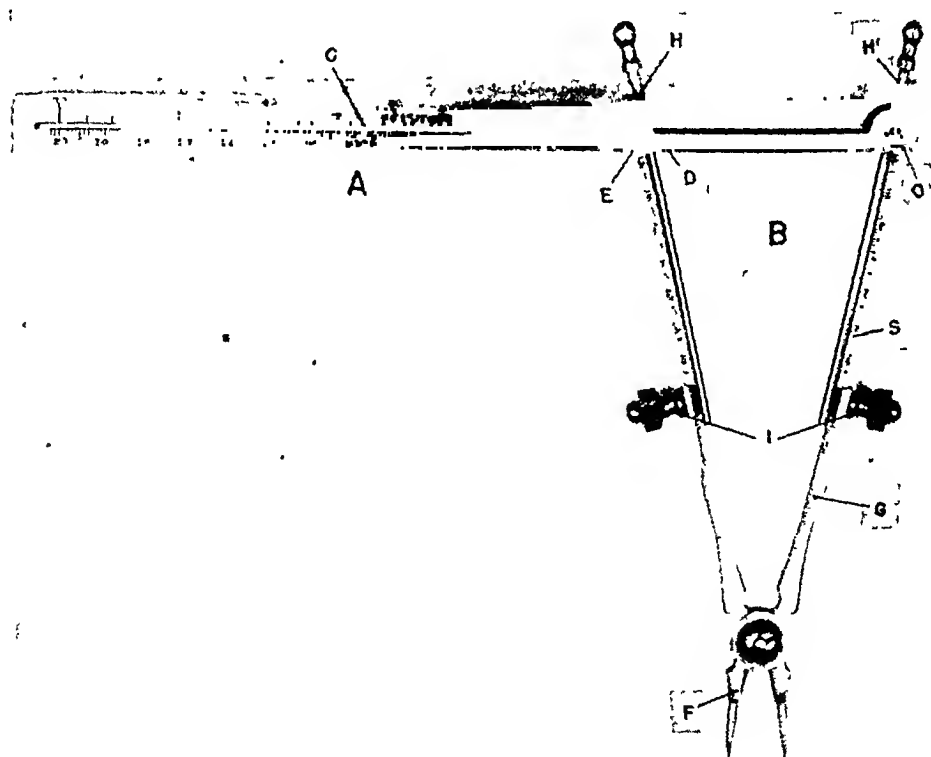


Fig. 2.

SUMMARY

A new caliper calculator for determining heart rate and cycle length in the routine measurement of electrocardiograms is described. The method of its use is outlined, and its advantages for quick calculations are discussed.

We wish to express appreciation to Dr. L. N. Katz, for his guidance in the development of this instrument.

THE DETERMINATION OF URINE UROBILINOGEN

THE INTERPRETATION OF THE RATE OF COLOR DEVELOPMENT OF THE EHRlich REACTION

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CHARLES S. DAVIDSON, M.D., C.M., BOSTON, MASS.

INTRODUCTION

WIDESPREAD recognition of the value of a clinical laboratory test for urobilinogen in the study and diagnosis of liver disease and jaundice has led to the development of a number of methods for the determination of urobilinogen in the urine and stool. The diagnostic value of a purely qualitative test is limited; consequently, efforts have been made to develop a satisfactory quantitative method.

Wallace and Diamond¹ introduced a method involving progressive dilutions of urine. Ehrlich's reagent (paradimethylaminobenzaldehyde in a solution of hydrochloric acid) is added to a series of tubes containing progressive dilutions of urine, and the tube of highest dilution showing evidence of a reddish-pink color is taken as an expression of the quantity of urobilinogen in the urine sample. Five minutes are allowed after the addition of Ehrlich's reagent for production of the "maximum intensity" of color, for it was observed that more color was produced if the reaction was allowed to proceed for some time before readings were taken.

Watson²⁻⁴ has presented a modification of Terwen's⁵ extraction method for the quantitative determination of urobilinogen in the urine and stool. This method is the most satisfactory for accurately determining urobilinogen, but it is time consuming and not adaptable to routine clinical use. In recognition of this, Watson⁶ recently described a simplified procedure in which color is produced in urine by the addition of Ehrlich's reagent followed by a saturated solution of sodium acetate. The intensity of color is then visually compared with dye standards or measured in the Evelyn photoelectric colorimeter. The solution of sodium acetate both causes maximum development of color and brings the color-producing aldehyde-condensation reaction to a halt. Following the addition of the sodium acetate, the color produced begins to fade gradually, but this is negligible if the colorimeter reading is made within five minutes. Watson points out that in addition to urobilinogen the simplified method also measures other substances in urine which will produce a reddish color when treated with Ehrlich's reagent. Indole and skatole are two of these substances, but there are others about which little is known. Watson, however, states ". . . these substances are usually present in amounts roughly proportional to that of urobilinogen, so that from the clinical standpoint the information gained is usually

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equivalent to that obtained with the exact quantitative method (petroleum ether extraction procedure)."

In the simple direct method as Watson⁶ describes it, the immediate addition of sodium acetate to the mixture of urine and Ehrlich's reagent is implied. In some laboratories in which this method is used, it has been customary to modify Watson's procedure by allowing a time interval to elapse between the addition of Ehrlich's reagent and the sodium acetate solution to assure maximum color development. The experiments outlined in the present report suggest that such a modification of Watson's method is not justified and, indeed, that the time interval should be standardized at fifteen seconds.

METHODS AND MATERIALS

Two methods described by Watson (the petroleum ether extraction method⁴ and the simplified direct method⁶) were used with the modifications to be described. The material consisted of twelve urine samples from normal individuals and nine urine samples from patients in whom increased amounts of urobilinogen were expected. The latter were all patients with Laënnec's cirrhosis of the liver, with the exception of one patient with infectious hepatitis in early convalescence. All the urine specimens were single voided specimens not accurately representing any constant period of excretion and obtained both in the late morning and in the early afternoon. On all of these urine specimens the simple direct method for the determination of urobilinogen, described by Watson,⁶ utilizing the Evelyn photoelectric colorimeter, was used; in addition, the factor of time was studied, readings being made at thirty seconds and five, ten, twenty, and thirty minutes between the time of adding Ehrlich's reagent and the sodium acetate solution. For the sake of standardization, all readings were made thirty seconds after the sodium acetate solution was added and the solutions mixed. In addition, out of the group of nine "abnormal" urine samples five determinations of urobilinogen were carried out by the ether extraction method, described by Watson,⁴ utilizing the Evelyn photoelectric colorimeter. This was likewise modified to include the factor of time. After the addition of Ehrlich's reagent to the petroleum ether which contains the extracted urobilinogen and after the extraction of the urobilinogen from the ether by agitation, the Ehrlich fraction was separated and divided into three fractions to which the sodium acetate solution was added at intervals of five, ten, and thirty minutes from the time of adding Ehrlich's reagent to the ether extract. In those urines on which both methods were used, the procedures were carried out simultaneously.

RESULTS

The results are shown in Figs. 1, 2, and 3. The intensity of the color, which develops in urine before the reaction with Ehrlich's reagent is stopped by the addition of sodium acetate solution (interpreted as Ehrlich units per 100 c.e.), is plotted against the time interval between the addition of Ehrlich's reagent and sodium acetate to the urine. In Fig. 1 the curves are those obtained, using the simple method, from the urine of normal individuals; those in Fig. 2

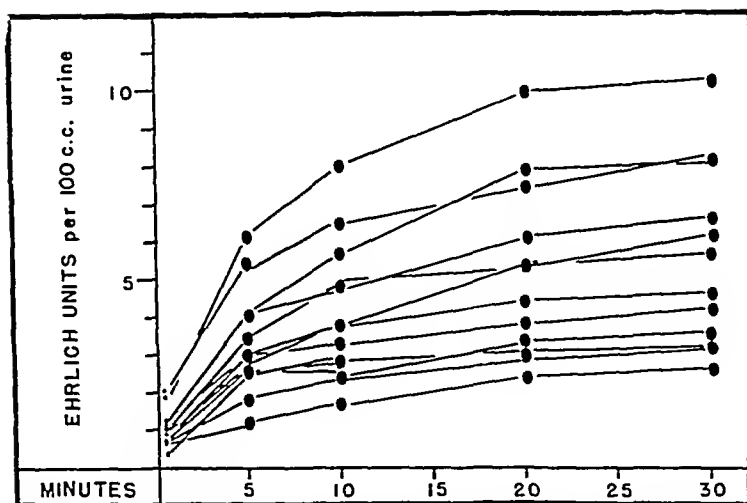


Fig. 1.—Ehrlich reaction in normal urine at various time intervals before addition of sodium acetate.

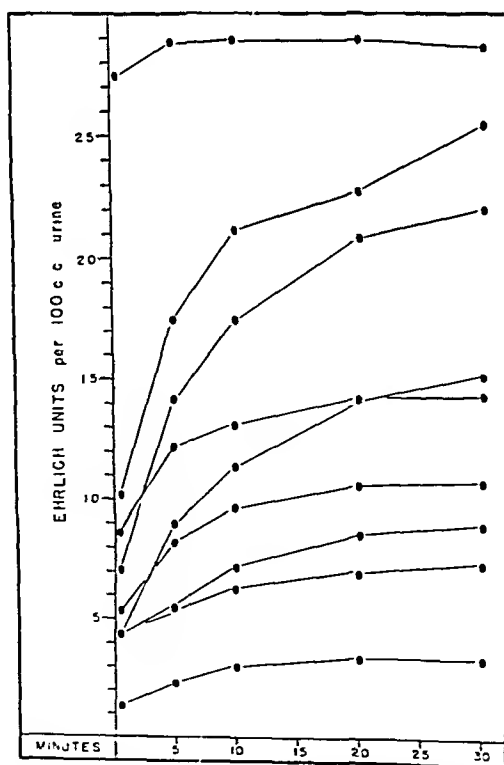


Fig. 2.—Ehrlich reaction in urine from patients with liver disease at various time intervals before addition of sodium acetate.

are from the urines of the patients with liver disease; and those in Fig. 3 are the plotted values obtained when both the simple method and the extraction method were done on four different urine samples.

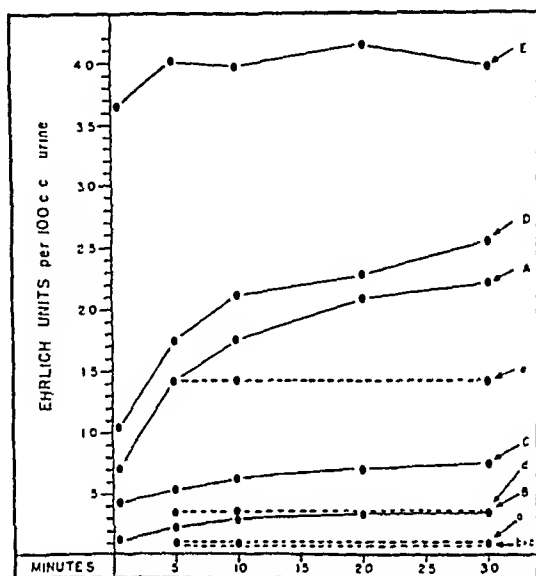


Fig. 3.—Comparison of extraction and simple method for determination of urine urobilinogen.

DISCUSSION

The results indicate that the intensity of color produced in urine after the addition of Ehrlich's reagent will increase with the length of time elapsing between the addition of Ehrlich's reagent and the addition of the sodium acetate solution. This increase in intensity tends to be most rapid in the first ten minutes, thereafter slowing down, and in most cases is negligible at thirty minutes. These data also show that the extracted urobilinogen does not participate in this increase in color intensity. As seen in the curves obtained from both the "normal" urines and the "abnormal" urines, the initial intensity of color bears no constant relation to the maximum intensity developed in thirty minutes. With increasing time between the addition of Ehrlich's reagent and of the sodium acetate solution, a wide variation in the intensity of color produced occurs which makes the distinction between the normal and abnormal urines progressively less marked. Thus, ideally, the sodium acetate should be added immediately after the Ehrlich's reagent; the time at which to read the color produced is as close to zero time as can be done. The values shown for the normals at thirty seconds would indicate that the upper limit of normal expressed in Ehrlich units per 100 c.c. of urine is more than double that suggested by Watson, but if the values are extrapolated back to zero time they would practically all fall within the upper limit of 1.0 Ehrlich unit per 100 c.c. of urine, as Watson indicates. As it is impossible to make readings at zero time,

fifteen seconds has been chosen as the most practical time. Fig. 3 suggests that perhaps the increase in color is not proportional to the amount of urobilinogen present in the urine. Whether this is a major criticism of the method or not cannot be determined from the limited data presented, but it would suggest that further investigation along this line is necessary to establish more accurately the normals for the simple direct method and the relationship if any to the actual amount of urobilinogen present in the urine specimen.

SUMMARY

When Ehrlich's reagent is added to urine, the color produced increases with time. Extracted urobilinogen promptly develops full color with Ehrlich's reagent, which suggests that subsequent increases in color with time are due to substances other than urobilinogen. In liver disease the immediate reaction appears to be the most significant. It is, therefore, recommended that the reaction between Ehrlich's reagent and urine be stopped (by the addition of saturated sodium acetate solution) in as short a time as is convenient and that can be standardized. Exactly fifteen seconds appears to be the most satisfactory time interval.

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pedic Surgeons; Registry of Veterinary Pathology, established in 1944, sponsored by the American Veterinary Medical Association; and Registry of Gerontology, established in 1945, sponsored by the Gerontological Society, Inc.

Plans for additional registries are under consideration. A professional scientific society wishing to sponsor a registry should communicate with the Director, Army Institute of Pathology, 7th Street and Independence Ave, S.W., Washington 25, D. C. The society appoints a committee to work with the Director in supervision of the activities of the Registry and makes an annual contribution to the budget which is administered by the National Academy of Sciences.

All specimens in the Registry are available for review and research by competent investigators. Sets of slides and accompanying syllabuses on special fields are available for loan to the civilian professions and officers in the federal services. Physician, dentists, and veterinarians are urged to send unusual specimens together with an abstract of the history to the Registry. The contributor receives a report on each specimen and is asked to keep the Registry informed of the follow-up on the patient.

With the reorganization of the Army Institute of Pathology to be completed during 1946 and 1947, a full-time scientific director of the American Registry of Pathology will be appointed, and sufficient clerks and technicians will be available to assure adequate use of the registries for diagnosis, research, training of young men, and education of the professions.

DEMONSTRATION OF RH ANTIBODIES IN THE NEWBORN AND FURTHER EVIDENCE OF THE PATHOGENESIS OF ERYTHROBLASTOSIS

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DALLAS, TEXAS

IT IS generally accepted that hemolytic disease of the newborn, or erythroblastosis, with a few possible exceptions is caused by the production of Rh antibodies in Rh-negative mothers bearing Rh-positive children. The present concept of the pathogenesis of this disease is based upon the reports of Levine and Stetson,¹ Landsteiner and Wiener,² Levine and associates,³⁻⁵ Wiener and co-workers,^{6, 7} Witebsky and Heide,⁸ and others. If the concept of Forbus⁹ that disease is reaction to injury is applied to erythroblastosis, it would seem desirable to attempt to establish the chain of evidence demonstrating the source and nature of the injury and the reaction to it. Although the evidence for each step in the pathogenesis of the disease is not complete, the sequence of events may be outlined as follows: (1) The Rh antigen in the fetal erythrocytes inherited from the father (2) gains access to the mother's circulation, (3) evoking a maternal response of Rh antibodies (4) which, in turn, pass across the placenta into the fetal circulation or, after birth, are absorbed from the mother's milk and (5) are adsorbed upon the infant's red cells, (6) resulting in their destruction, to produce anemia and jaundice. Further reactions on the part of the tissues of the newborn, such as myeloid hyperplasia, ectopic hematopoiesis, and edema, then follow, according to the severity of the disease. While the previously mentioned steps are commonly accepted, it should be noted that number 2, the mode of access of fetal cells to the maternal circulation, probably cannot be directly determined. However, Levine¹⁰ has shown by analogy how minute quantities of human red cells may immunize rabbits and suggests that comparable numbers of erythrocytes might easily enter the maternal circulation through the slight placental defects known to exist. Furthermore, the production of antibodies in the mother is in itself evidence of the introduction of antigen of the type present in the fetal cells.

In this report we are concerned principally with the demonstration of evidence for events 4, 5, and 6 in the pathogenesis of erythroblastosis as outlined. This evidence consists of the identification of Rh antibodies in fetal serum, their specific adsorption on the cells of the newborn, and the hemolytic destruction of Rh-positive human erythrocytes *in vitro*. The demonstration of Rh antibodies in the infant has been difficult and uncertain, although Haberman and Hill¹¹ in 1944 first described the finding of Rh agglutinins adsorbed on the erythrocytes

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BOOK REVIEWS

Everyday Psychiatry. By *John D. Campbell, M.D.*, Commander, M.C., U.S.N.R., Chief Neuropsychiatrist, U. S. Naval Base Hospital No. 5; formerly Chief Neuropsychiatrist, U. S. Naval Hospital, Charleston, S. C., and Visiting Lecturer in Psychiatry, Medical College of S. C.; Diplomate, American Board of Neurology and Psychiatry. J. B. Lippincott Company, Philadelphia, 1945. Price \$6.00. Cloth with 333 pages.

The author's aim is to present psychiatric medicine to physicians as to make it as naturally acceptable and usable by them as internal medicine, neurologic medicine, gynecologic medicine, or surgical medicine.

To achieve his aim, a simple practical scheme is required for describing and evaluating personality and the common psychiatric disorders. The author presents such a scheme, which involves four "basic personality traits" and two "secondary personality factors." The four basic personality factors are intelligence, conscience, emotional reaction, and psychosexual development. The two secondary personality factors are sociability and special modes of adjustment. Abnormal personalities are ranked (very crudely) on each of these six scales, and when so ranked group themselves into the standard patterns of borderline (nonpsychotic) psychiatric disorder: mental deficiency, psychopathic personality, psychoneurosis and subdivisions, homosexuality, schizoid personality, cycloid personality, chronic alcoholism. These patterns are described in concise, clear, vigorous language, with excellent life histories as illustrations.

In general, the author has succeeded in his task. The physician who uses the book will lose much of his insecurity and anxiety about everyday psychiatric problems. Further, as he faces these problems his interest and skill will increase with resulting benefit to medical practice as well as psychiatry.

There is one regrettable feature of this otherwise very useful book. The author gives the impression (though he denies this as his intention) that the only proper goal of psychiatric treatment is adjusting the patient to his constitutionally given, quantitatively fixed properties or personality factors. This impression ought not to be given for two reasons: (1) Estimation of a patient's properties, by the author's method, is extremely crude, and can be done only within such broad limits as to make meaningless too assured statements about a patient's "constitutional limitations"; (2) experienced psychotherapists see quantitative changes take place in the behavior of patients under treatment; such changes are essential to the success of treatment. It must be said in fairness to the author, however, that no adequate scientific demonstration of their occurrence has yet been made.

GEORGE SASLOW, M.D.

ANNOUNCEMENT

Army Institute of Pathology and American Registry of Pathology

What is now known as the Army Institute of Pathology was established in 1863 as the Army Medical Museum. During World War II the activities of the Institute were greatly expanded, especially in the field of diagnostic pathology and research. There are now on file over 170,000 accessions. The results of research at the Institute during the past few years will be published in a volume of about fourteen hundred pages as a part of the official history of World War II. The present director is Colonel J. Earl Ash, who will be succeeded on October 1 by Colonel Raymond O. Dart.

On request of Major General Norman T. Kirk, Surgeon General of the Army, the Committee on Pathology of the National Research Council, Division of Medical Sciences, in late 1945 prepared a report on the future development of the Institute. The report has been approved by the Surgeon General and by the War Department.

The essential recommendations in this report are: (1) that a new building of adequate size be constructed; (2) that the Institute be organized in four divisions (Department of Pathology, Army Medical Illustration Service, Army Medical Museum, and American Registry of Pathology), each headed by a competent specialist; (3) that the staff of the Institute be drawn from both the commissioned ranks of the Army and from the civilian professions; (4) that a comprehensive educational and training program be undertaken; (5) that the vast store of material at the Institute be used for research; and (6) that the services in pathology in the veteran's hospitals be centralized at the Institute.

The American Registry of Pathology founded in 1922 thus is, and will continue to be, an integral part of the Army Institute of Pathology. There were, on Jan. 1, 1946, over 43,000 cases registered. To effectuate the new plans as they relate to the Registry, the National Research Council, Division of Medical Sciences, appointed a Committee on the American Registry of Pathology. The members of the Committee are Howard T. Karsner, Chairman, Cleveland; Colonel J. E. Ash, Washington; Brigadier General George R. Callender, Washington; Colonel Baldwin Lueke, Philadelphia; Robert A. Moore, St. Louis; Benjamin Rones, Washington; A. R. Shands, Jr., Wilmington; and Henry A. Swanson, Washington.

At the present time there are fourteen registries as a part of the American Registry of Pathology. These include Registry of Ophthalmic Pathology, established in 1922, sponsored by the American Academy of Ophthalmology and Otolaryngology; Lymphatic Tumor Registry, established in 1925, sponsored by the American Association of Pathologists and Bacteriologists; Bladder Tumor Registry, established in 1927, Kidney Tumor Registry, established in 1940, and Prostatic Tumor Registry, established in 1943, sponsored by the American Urological Association; Registry of Dental and Oral Pathology, established in 1933, sponsored by the American Dental Association; Registry of Otolaryngological Pathology, established in 1935, sponsored by the American Academy of Ophthalmology and Otolaryngology; General Tumor Registry, established in 1937, sponsored by the American Society of Clinical Pathologists; Registry of Dermal Pathology, established in 1938, sponsored by the American Academy of Dermatology and Syphilology; Chest Tumor Registry, established in 1942, sponsored by the American Society of Thoracic Surgeons; Registry of Neuropathology, established in 1943, sponsored by the American Association of Neuropathologists; Registry of Orthopedic Pathology, established in 1943, sponsored by the American Academy of Ortho-

pedic Surgeons; Registry of Veterinary Pathology, established in 1944, sponsored by the American Veterinary Medical Association; and Registry of Gerontology, established in 1945, sponsored by the Gerontological Society, Inc.

Plans for additional registries are under consideration. A professional scientific society wishing to sponsor a registry should communicate with the Director, Army Institute of Pathology, 7th Street and Independence Ave, S.W., Washington 25, D. C. The society appoints a committee to work with the Director in supervision of the activities of the Registry and makes an annual contribution to the budget which is administered by the National Academy of Sciences.

All specimens in the Registry are available for review and research by competent investigators. Sets of slides and accompanying syllabuses on special fields are available for loan to the civilian professions and officers in the federal services. Physician, dentists, and veterinarians are urged to send unusual specimens together with an abstract of the history to the Registry. The contributor receives a report on each specimen and is asked to keep the Registry informed of the follow-up on the patient.

With the reorganization of the Army Institute of Pathology to be completed during 1946 and 1947, a full-time scientific director of the American Registry of Pathology will be appointed, and sufficient clerks and technicians will be available to assure adequate use of the registries for diagnosis, research, training of young men, and education of the professions.

DEMONSTRATION OF RH ANTIBODIES IN THE NEWBORN AND FURTHER EVIDENCE OF THE PATHOGENESIS OF ERYTHROBLASTOSIS

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IT IS generally accepted that hemolytic disease of the newborn, or erythroblastosis, with a few possible exceptions is caused by the production of Rh antibodies in Rh-negative mothers bearing Rh-positive children. The present concept of the pathogenesis of this disease is based upon the reports of Levine and Stetson,¹ Landsteiner and Wiener,² Levine and associates,³⁻⁵ Wiener and co-workers,^{6,7} Witebsky and Heide,⁸ and others. If the concept of Forbus⁹ that disease is reaction to injury is applied to erythroblastosis, it would seem desirable to attempt to establish the chain of evidence demonstrating the source and nature of the injury and the reaction to it. Although the evidence for each step in the pathogenesis of the disease is not complete, the sequence of events may be outlined as follows: (1) The Rh antigen in the fetal erythrocytes inherited from the father (2) gains access to the mother's circulation, (3) evoking a maternal response of Rh antibodies (4) which, in turn, pass across the placenta into the fetal circulation or, after birth, are absorbed from the mother's milk and (5) are adsorbed upon the infant's red cells, (6) resulting in their destruction, to produce anemia and jaundice. Further reactions on the part of the tissues of the newborn, such as myeloid hyperplasia, ectopic hematopoiesis, and edema, then follow, according to the severity of the disease. While the previously mentioned steps are commonly accepted, it should be noted that number 2, the mode of access of fetal cells to the maternal circulation, probably cannot be directly determined. However, Levine¹⁰ has shown by analogy how minute quantities of human red cells may immunize rabbits and suggests that comparable numbers of erythrocytes might easily enter the maternal circulation through the slight placental defects known to exist. Furthermore, the production of antibodies in the mother is in itself evidence of the introduction of antigen of the type present in the fetal cells.

In this report we are concerned principally with the demonstration of evidence for events 4, 5, and 6 in the pathogenesis of erythroblastosis as outlined. This evidence consists of the identification of Rh antibodies in fetal serum, their specific adsorption on the cells of the newborn, and the hemolytic destruction of Rh-positive human erythrocytes in vitro. The demonstration of Rh antibodies in the infant has been difficult and uncertain, although Haberman and Hill¹¹ in 1944 first described the finding of Rh agglutinin adsorbed on the erythrocytes

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of the newborn as well as free in the serum of cord blood. The adsorbed anti-Rh agglutinins were successfully demonstrated by the elution technique of Landsteiner and Miller¹² in which the agglutinins were removed from the red cells into saline solution by brief subjection to 56° centigrade. This method of demonstrating adsorbed Rh agglutinins has recently been confirmed by Carter and Loughrey.¹³ Although we have employed the elution technique routinely in all cases of hemolytic disease of the newborn since our first report, positive tests have been found in only three out of a total of ten cases of erythroblastosis. The presence of antibody adsorbed on the Rh-positive erythrocytes of the infant was never demonstrated except when also present free in the infant's serum and in the serum of the mother. The use of the blocking test slightly increased the number of positive demonstrations of fetal antibodies as shown in Case 2 (eluate) and in Case 3 (in vivo blocking).

A new approach to this problem of detecting fetal antibodies is the use of antihuman globulin serum. Coombs, Mourant, and Race^{14, 15} have described the successful use of such a serum for the detection of weak and incomplete or blocking antibodies in various sera from isoimmunized individuals and suggested it might be employed to identify fetal antibodies not otherwise demonstrable. In a recent paper the same authors¹⁶ reported on the actual use of their method to detect in vivo isosensitization of red cells in babies with hemolytic disease. The action of the antihuman globulin serum is to develop an observable agglutination where the specifically adsorbed antibodies are not able to do so by themselves (blocking or incomplete antibodies). Because of the analogy to the photographic development of the visible image, for convenience we have applied the descriptive term "developing test" to the use of the antihuman globulin serum to develop an obvious agglutination reaction. In our routine service we have employed this "developing" serum both as a test for weak antibodies in the serum of mothers and as a diagnostic test for hemolytic disease of the newborn.

METHODS

Testing of the serum of mother and infant was done, in all cases where isoimmunization was suspected, by the test tube method of Levine and Stetson.¹ Wiener's method was used to detect the incomplete or blocking antibody described by Race¹⁷ and Wiener¹⁸ as soon as these reports were available. The slide test of Diamond and Abelson¹⁹ and the conglutination test of Wiener²⁰ were used in some studies but were not employed routinely.

Elution Test.—The elution technique of Landsteiner and Miller has been used routinely to attempt to demonstrate adsorbed Rh agglutinins on the cells of the blood of all cases of hemolytic disease of the newborn and on the erythrocytes of all babies whose mothers showed a positive test for Rh antibodies. The red corpuscles of cord blood were washed three times in cold saline, thrown down by centrifugation, and, after removing the saline the third time, 1 c.c. of fresh saline was added to a 0.25 c.c. aliquot of packed cells. After resuspension these cells were subjected to 56° C. in a water bath for five minutes. At the end of this period the suspension was immediately spun at 3,000 R.P.M. for

five minutes keeping the temperature at 56° C. with hot water in the centrifuge cups. The supernatant saline eluate was removed and recentrifuged to insure removal of all infant cells. This hemoglobin tinted fluid was then used with known Rh-positive cells by mixing one drop of the eluate with one drop of 2 per cent suspension of erythrocytes. After one hour incubation at 37° C. the tube was centrifuged gently and examined for agglutination.

Blocking Test.—If no clumping was seen in the agglutination test, one drop of anti-Rh serum of known potency was added to each tube and after a second incubation of one hour observed for agglutination. Lack of agglutination after this addition of known Rh antibody indicated presence of blocking or incomplete agglutinins.

Developing Test.—For the purpose of detecting weak Rh agglutinins, incomplete antibodies, or immune globulin, an antihuman globulin serum as described by Coombs, Mourant, and Race¹⁵ was used. If the routine test for agglutinins using known Rh-positive cells was negative, the red cells of this suspension were washed three times with saline to remove mechanically adherent serum proteins. One drop of the developing serum was then added to one drop of the suspension of washed Rh-positive erythrocytes. Following the incubation for one hour at 37° C. the tubes were centrifuged lightly and observed for agglutination. A positive developing test indicating specifically adsorbed immune globulin was shown by definite clumping of the red cells.

Developing Test for Sensitized Erythrocytes.—As a possible diagnostic test for erythroblastosis, the developing serum was used to demonstrate the presence of antibody specifically adsorbed on the red cells in vivo. The erythrocytes of suspected cases of hemolytic disease of the newborn were washed three times to remove mechanically adherent globulin and then suspended in saline. One drop of developing serum was added to one drop of 2 per cent suspension of these washed erythrocytes and incubated for one hour at 37° centigrade. The tubes were then centrifuged at 500 R.P.M. for one minute and examined for agglutination. As a control, one drop of developing serum was added to a 2 per cent suspension of washed known Rh-positive cells previously suspended in serum containing known incomplete Rh antibodies. A second control using Rh-negative erythrocytes treated in a similar manner was used. Initially a third control consisting of a drop of washed Rh-positive cells, not previously sensitized, plus a drop of developing serum was also employed.

Demonstration of Hemolytic Activity of Rh Serum in Vitro.—To 3 c.c. of freshly drawn heparinized Rh-positive whole blood was added 0.3 c.c. of anti-Rh serum having a titer of 1:20,000 resulting in an effective final titer of 1:2,000. In order to obtain the net hemolysis due to the action solely of the Rh serum, it was necessary to set up reagent blanks and a suitable control. The first blank consisted of 0.3 c.c. of the anti-Rh serum and 3 c.c. of normal saline to make a final volume equal to that of the test and was used for the purpose of determining the amount of hemoglobin already present in the anti-Rh serum used in the experiment. The second blank was made up of 3 c.c. of saline and 0.3 c.c. of the normal serum used in the control in place of the anti-Rh serum

used in the experiment. The control set up for the purpose of determining the amount of hemoglobin released by normal heparinized blood without anti-serum consisted of 3 c.c. of the same heparinized blood used in the experiment plus 0.3 c.c. of the normal serum used in the second blank.

In order to eliminate the possible hemolytic effect of mechanical action or introduction of bacterial contaminants, all the blood used in the experiment was drawn through a No. 19 needle into a dry 50 c.c. syringe and after removal of the needle gently expelled into a sterile 125 c.c. flask containing 0.2 c.c. of sterile heparin solution (1,000 units per c.c.) and gently rotated. Three cubic centimeter aliquots were immediately added to each of twelve sterile test tubes, the first six of which contained 0.3 c.c. of the 1:20,000 titer anti-Rh serum and the second, or control, series of six tubes contained only 0.3 c.c. of normal serum.

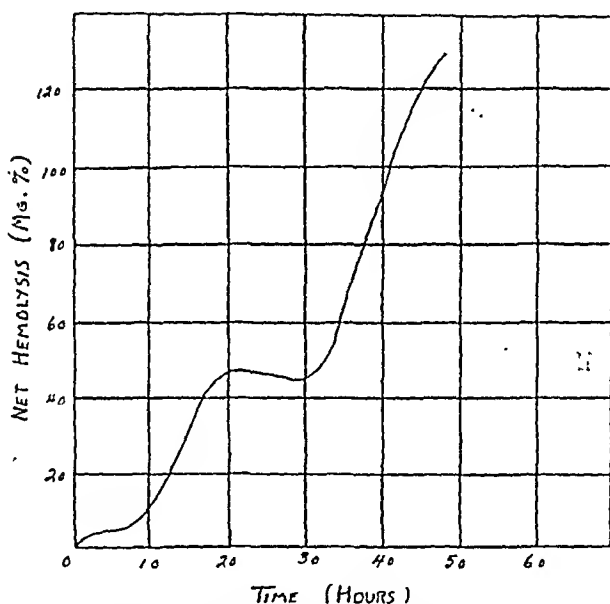


Fig. 1.—The hemolytic action of anti-Rh serum (1:2,000 titer) on heparinized Rh-positive whole blood, plotted as net hemolysis (mg. % hemoglobin) after deducting hemoglobin released in control blood not treated with anti-Rh serum.

After mixture, one tube from the experiment and one from the control series were immediately centrifuged and the supernatant cell-free fluid analyzed for the hemoglobin content by a modification of the Bing and Baker^{21*} quantitative test for hemoglobin. The remaining ten tubes were incubated at 37.5° C. and one tube from each series withdrawn at 2, 6, 20, 30, and 48 hours for analysis of the hemoglobin content of the supernatant cell-free plasma. The color reactions were read in a Lumetron two-cell photoelectric colorimeter using narrow band filters and a high sensitivity double reflecting galvanometer.

*The modification consists essentially of the use of highly purified reagent grade benzidine dihydrochloride (Merek & Company, Inc., New York, N. Y.) in place of the cruder benzidine base for the benzidine reagent. The use of this product eliminates the necessity of purification with charcoal, since a blank using the dihydrochloride produces no appreciable color and if stored in an icebox, it maintains its effectiveness for a period of days.

Net hemolysis was calculated in mg. of hemoglobin from the formula
 $(H_t - b_t) - (H_c - b_c) = \text{net hemolysis.}$

Where

H_t = Mg. % of hemoglobin in supernatant of tubes containing blood and antiserum (experiment).

H_c = Mg. % of hemoglobin in supernatant of tubes containing blood and normal serum.

b_t = Mg. % of hemoglobin in first blank (Rh serum + saline).

b_c = Mg. % of hemoglobin in second blank (normal serum + saline).

The results are shown in Table II and represented graphically in Fig. 1.

RESULTS

CASE 1.—O. M. was a severely icteric full-term Rh-positive male infant with enlarged liver and spleen and with erythroblasts and normoblasts in the blood. The Rh-negative mother had previously given birth to two Rh-positive children. The first was normal and still living; the second, icteric at birth, had symptoms suggestive of kernicterus and died at the age of 1 year. Before delivery the mother's serum showed Rh agglutinins with a titer of 1:20 for Rh₁' red cells and 1:40 Rh₂' cells. After delivery the mother's serum showed 1:64 and 1:256 titers for the same respective Rh-positive erythrocytes. The infant's cord serum showed positive agglutination against Rh₁' cells but not against Rh₂'. However, the eluate from the infant's erythrocytes contained antibodies which agglutinated both Rh₁' and Rh₂'. A transfusion was started one hour after birth (100 c.c.), and 50 c.c. transfusions were given daily thereafter for a week. The child is now living and well.

Diagnosis: Severe hemolytic disease of the newborn.

CASE 2.—H. R. was an icteric full-term Rh-positive male infant with erythroblasts and normoblasts in the blood film. The Rh-negative mother had two normal Rh-positive children living and well. The mother's serum after delivery showed a 1:4 titer against Rh₁' and Rh₂' erythrocytes and a blocking antibody to 1:2. The infant's cord serum showed a 1:2 titer against Rh-positive cells, but no blocking antibody was demonstrated. The eluate of the infant's red cells showed very weak agglutinins and blocking antibodies against Rh₁' and Rh₂' cells. Complete recovery followed transfusions of Rh-negative blood. The child is now living and well.

Diagnosis: Moderately severe erythroblastosis.

CASE 3.—K. D., a white, Rh-positive, female, full-term infant apparently was normal at birth and turned icteric on the second day. The Rh-negative mother had had one former child which died shortly after labor. There were no previous abortions, miscarriages, or transfusions. The mother's undiluted serum showed agglutination of Rh-positive cells and a positive blocking test at a dilution of 1:16. The cord blood was not available for elution since this was an outside case. However, the infant's red cells typed Rh negative on the first day. On the second day a weak positive reaction was obtained using the same Rh-typing serum. On the third day the child was clearly positive, thus demonstrating that in vivo blocking antibody was present on the infant's erythrocytes at birth. Blood films showed progressive increase of erythroblasts and normoblasts with a maximum of 59 erythroblasts and 12 normoblasts per 100 leucocytes on the third day. The child was taken off the breast, and transfusions were started on that day. At the age of 1 year it was reported that the child did not have normal control of muscles.

Diagnosis: Severe hemolytic disease of the newborn with kernicterus.

CASE 4.—E. K., a white, full-term, normal-appearing, Rh-positive male infant developed moderate icterus on the second day. This jaundice cleared up partially by the time the baby was discharged. The erythrocyte count was 6,100,000 per cubic millimeter the first day and 5,600,000 on the sixth. In the blood film 2 normoblasts per 100 leucocytes

were seen on the second day but none thereafter. The Rh-negative mother showed agglutinating antibodies at a titer of 1:32, blocking antibodies at 1:2, and a positive developing test at 1:32. The two previous children ages 7 and 3, were living and well. The infant showed agglutinating antibodies in the undiluted cord blood serum. No blocking antibodies were found. The developing test was not performed on the infant's blood. No transfusions were given.

Diagnosis: Mild hemolytic disease of the newborn.

CASE 5.—R. V., a white, full-term Rh-positive infant was stillborn. The mother had had four previous pregnancies: the first was stillborn prematurely; the second having erythroblastosis was transfused and is now living; and the third and fourth pregnancies terminated in abortions. After the first miscarriage the mother had received several transfusions. The mother's serum showed an agglutinating titer of 1:64 against both Rh₀' and Rh₀' erythrocytes, no blocking antibody, and a titer of 1:64 by the developing test performed months later on serum preserved by freezing. Agglutination and blocking tests on the cord serum were negative, but a positive result was obtained on the undiluted cord serum with the developing test even though the cord serum had been preserved six months by freezing.

Diagnosis: Severe hemolytic disease of the newborn with fetal death.

CASE 6.—W. C., a white Rh-positive female infant, two weeks premature, appeared normal at birth but developed mild jaundice on the second day which cleared by the time of discharge on the eighth day. Clinically this was considered to be physiologic icterus. By a former husband the Rh-negative mother had had one child which had died of pneumonia at ten months. The mother's serum showed agglutinating antibodies at 1:2 dilution, no blocking antibodies, and developing antibodies at 1:64 dilution. The infant's serum was negative to agglutinating, blocking, and developing tests for antibodies. However, the infant's washed erythrocytes gave a 4 plus agglutination with developing serum. After elution these red cells failed to clump with the same antihuman globulin serum. Nevertheless the antibodies in the eluate could not be detected even with known Rh-positive cells and developing serum. The hemoglobin was 20.15 Gm. per 100 c.c. on the third day and 14.14 Gm. on the sixth. The child was taken home on the eighth day but became progressively paler. The infant's red cells still tested 3 plus with developing serum on the nineteenth day. As a result of this serologic evidence of adsorbed hemolytic antibody still present on the erythrocytes, the baby was taken off the breast and admitted to the hospital. On the twenty-eighth day the erythrocyte count was 2,300,000, hemoglobin 6.6 Gm., and the blood film showed anisocytosis, stippling, and one normoblast. At this time the infant's washed red cells still showed a 2 plus reaction with developing serum. Two transfusions of 100 c.c. each were given, and the child was discharged in good condition. At thirty-six days the washed red cells gave a negative test with the developing serum.

Diagnosis: Mild hemolytic disease of the newborn due to Rh isoimmunization aggravated by breast feeding. Recovery with transfusion after removal from the breast.

CASE 7.—M. L., a white, male, Rh-positive full-term icteric infant had 14 per cent normoblasts and a red cell count of 4,000,000 on day of birth. The Rh-negative mother had a history of two previous pregnancies, with one child, 4 years old living and well and with one miscarriage at three months gestation since birth of the first child. No transfusions had been given. The mother's serum showed a negative test for agglutinating Rh antibodies but positive tests for blocking antibodies at 1:2 dilution and developing antibodies at 1:16. Infant's erythrocytes were not available for performance of the developing test. The baby was given a transfusion of 110 c.c. of blood on the second day which elevated the red cell count from 2,800,000 to over 3,000,000. On the fourth and twelfth days additional transfusions of about 100 c.c. each were given. No normoblasts were seen in the blood film after the third day, and the red count slowly rose to 4,200,000 on day of discharge. The infant had convulsions from the fourth day up to the twelfth day, the most severe seizure on the tenth

being accompanied with cyanosis. At last report, about one month later, the child looked well but had slightly over 2,000,000 red cells.

Diagnosis: Moderately severe hemolytic disease of the newborn.

CASE 8.—J. E. was a white, female, macerated stillborn infant, weighing 3 pounds, 11 ounces; the estimated period of gestation was eight months. The Rh-negative mother had received two transfusions following an appendectomy two years ago. There were no other pregnancies. The mother's serum showed agglutinating antibodies at 1:2 dilution, blocking antibodies at 1:32, and developing antibodies at 1:2048. Unfortunately, no specimens were obtained from the macerated edematous fetus.

Diagnosis: Hydropic, macerated fetus apparently due to Rh isoimmunization.

CASE 9.—W. S., a white, full-term, Rh-positive female infant was apparently normal at birth but turned icteric on the first day. The Rh-negative mother had one previous normal child now living and well. There was no history of transfusions or other pregnancies. The mother's serum showed an agglutinating titer of 1:128 for Rh-positive cells, a negative blocking test, and a positive developing test at a titer of 1:128. The infant's serum showed negative agglutinating and blocking tests but a positive developing test with the serum undiluted. The baby's washed erythrocytes, however, showed a 4 plus reaction with developing serum. Furthermore, the eluate from the infant's red cells showed a positive agglutinating test at 1:4 dilution and a positive developing test at 1:8. No blocking antibodies were found. On the second day after birth the erythrocyte count was 3,456,000 with 15 normoblasts and 12 erythroblasts. Seventy-five cubic centimeters of Rh-negative blood were given on the second, 60 c.c. on the fourth, and 75 c.c. on the sixth day after birth. The child continued to remain jaundiced, stools were clay colored, and there was twitching and poor coordination of muscles. There was a loss of weight from 7 pounds, 15 ounces at birth to 6 pounds, 15 ounces at the seventeenth day. The red cell count at this time was 4,650,000 with 14.4 Gm. of hemoglobin.

Diagnosis: Severe hemolytic disease of the newborn with liver damage and possible kernicterus.

CASE 10.—W. L., a white, male, Rh-positive full-term infant was normal at birth and was discharged in good condition on the fifth day. No jaundice or other significant abnormality was noted at any time. The Rh-negative mother had received several transfusions during the three weeks of hospitalization following the delivery of her first child. The mother's serum showed no Rh antibodies by any of the three tests following the delivery of this second child. However, the infant's washed erythrocytes showed definite (2 plus) agglutination with developing serum. The eluate of the infant's red cells and the cord serum failed to show antibodies with any of the three tests, agglutination, blocking, or developing. Because of the definite developing test with the Rh-positive baby's erythrocytes it was concluded that antibodies in the mother's serum too weak to be detected had passed the placenta and had become concentrated on the fetal red cells. Accordingly, the infant was taken off breast feedings.

Diagnosis: Subclinical hemolytic disease of the newborn.

A summary of the serologic findings in the ten cases described is tabulated in Table I. The severity of the erythroblastosis, treatment, and outcome are also listed.

Since the developing test applied to erythrocytes of the newborn showed promise of being diagnostic for erythroblastosis, its specificity was investigated by performing routine developing tests on the erythrocytes of all cord bloods. A total of 365 developing tests on cord cells was done. Of these all tests were negative except erythroblastosis Cases 6, 8, and 9, previously described, together with Case 10 which did not show clinical erythroblastosis but which presented a background and history compatible with a subclinical isoimmuniza-

TABLE I

		RH ANTIBODIES IN SERUM			ANTI-BODY ADSORBED ON R.B.C.	ANTIBODY IN ELUATE FROM INFANT R.B.C.			SEVERITY OF ERYTHROBLASTOSIS	OUTCOME	TREATMENT
		AGGLUTINATING	BLOCKING	DEVELOPING	DEVELOPING TEST	AGGLUTINATING	BLOCKING	DEVELOPING			
Case 1	Mother	1:256	1:2	1:4†	*				Severe	Recovery	Early transfusions
	Infant	1:1	-	1:4†		1:4	-	1:4†			
Case 2	Mother	1:4	1:2	1:16	*				Moderately severe	Recovery	Early transfusions
	Infant	1:2	-	*	*	-	1:1	*	Severe	Kernicterus	Delayed transfusions
Case 3	Mother	1:1	1:16		*	*	*	*			
	Infant	*	In vivo	*	*	*	*	*			
Case 4	Mother	1:32	1:2	1:32†	*				Mild	Recovery	No transfusion
	Infant	1:1	-	*	**	1:1	*	*			
Case 5	Mother	1:64	-	1:64†	*				Severe	Stillborn	
	Infant	-	-	1:1	*	-	-	-			
Case 6	Mother	1:2	-	1:64					Moderate	Recovery	Off breast and transfused on nineteenth day
	Infant	-	-	-	++++	-	-	-			Transfusions begun on second day; no breast feeding
Case 7	Mother	-	1:2	1:16	*	*	*	*	Moderately severe	Partial recovery; still anemic	
	Infant	*	*	*	*	*	*	*			
Case 8	Mother	1:2	1:32	1:2048					Severe	Macerated fetus	
	Infant	*	*	*	*	*	*	*			
Case 9	Mother	1:128	-	1:128					Severe	Partial recovery; kernicterus	Delayed transfusion
	Infant	-	-	1:1	++++	1:4	-	1:8			
Case 10	Mother	-	-	-					Subclinical	Recovery	No treatment
	Infant	-	-	-	++	-	-	-			

*Test not done.

†Serum preserved several months in frozen state.

TABLE II.

TIME (HR.)	H _t HEMOGLOBIN IN RH-TREATED SAMPLE (MG. %)	b _t HEMOGLOBIN IN FIRST BLANK (RH SERUM + SALINE) (MG. %)	H _c HEMOGLOBIN IN UNTREATED SAMPLE (MG. %)	b _c HEMOGLOBIN IN SECOND BLANK (NORMAL SERUM + SALINE) (MG. %)	(H _t - b _t) - (H _c - b _c) = NET HEMOLYSIS IN RH-TREATED SAMPLE (MG. %)
0	6.2	2.2	7.7	5.4	1.7
2	8.0	2.2	8.4	5.4	2.8
6	9.2	2.2	8.5	5.4	3.9
20	73.0	2.2	29.4	5.4	46.8
30	102.1	2.2	60.0	5.4	45.3
48	222.4	2.2	92.5	5.4	133.1

tion. In this series negative developing tests were obtained on both sera and cells of five stillborn infants clinically not erythroblastosis. In addition there was one hydropic stillborn from which neither serum nor erythrocytes could be obtained (Case 8). Clinical icterus was found in four infants negative to the developing test, each of which had the same Rh type as the mother. These were considered to be examples of physiologic jaundice.

In Table II are presented the results of the study of hemolytic action of Rh antibodies *in vitro*. It should be noted that significant results such as are shown here could not be obtained when complement and Rh antiserum were added to a 5 per cent suspension of the same Rh-positive erythrocytes in normal saline.

DISCUSSION

The sequence of events in the pathogenesis of erythroblastosis as outlined in this paper are now generally accepted. However, many factors concerning the maternal antibody response, the concentration of these antibodies in the fetal circulation, their adsorption on fetal erythrocytes, and the mode of destruction of these corpuscles, had not been entirely clear. For example, the lack of correlation between the severity of the hemolytic reaction and the maternal antibody titer and even the lack of demonstrable agglutinins had been noted by several authors.²²⁻²⁵ It is believed that evidence is presented in this paper which sheds new light on some of these obscure phases of the erythroblastosis problem.

The successful removal of Rh antibodies from cord erythrocytes by elution in three cases of erythroblastosis is significant chiefly as a demonstration of the actual sensitization of the infant's cells preliminary to their destruction and as an opportunity to prove the specificity of this reaction by further readsorbing the eluted antibodies on known Rh-positive red cells. This readsorption may be indicated by agglutination or may require the blocking or developing tests, according to the type of Rh antibody involved (see Table I). This proof that the eluted material consisted of specific Rh antibodies of the same type found in the mother's serum indicates their origin in the mother as well as their transport across the placenta from the maternal circulation into that of the fetus. The specific nature of the union of the antibody to the Rh antigen of the erythrocyte is strongly suggestive of the destructive role this antibody must play in sensitizing the red cells for their hemolysis *in vivo* so characteristic of erythroblastosis. However, the actual hemolytic action of the Rh antibody *in vitro* had not been clearly demonstrated heretofore, although Diamond and Abelson¹⁹ had noted hemolysis in doing their slide test with a heavy suspension of red cells.

In Table II the actual hemolysis of Rh-positive erythrocytes *in vitro* by the Rh antibody is shown by the significantly greater liberation of hemoglobin as compared to controls. Further studies on the hemolytic activity of Rh antibodies *in vitro* already completed will be reported elsewhere. However, these experiments seem to afford evidence that these antibodies can act as hemolysins as well as agglutinins *in vitro*. In comparing the relatively weak

hemolysis seen *in vitro* with the severe hemolysis common *in vivo* (erythroblastosis), it should be noted that in the latter cases red cell destruction may be greatly enhanced by the action of the spleen in its role of phagocytosis of slightly damaged corpuscles. Finally, the titer of 1:2,000 effective in the test tube is sometimes exceeded in mother's serum, particularly if the developing test is used to demonstrate the titer of anti-Rh immune globulin.

While the elution technique, in conjunction with the demonstration of hemolysis *in vitro*, presents additional evidence for the sequence of events in the pathogenesis of erythroblastosis, it seems to offer little as a test to identify antibodies in the infant's blood. Only in those cases that showed antibodies in the serum of the mother were the elution tests on fetal cells positive as described previously by the present authors¹¹ and by Carter and Loughrey.¹² In the series of cases herein described it was further demonstrated that positive elution tests to demonstrate antibody adsorbed on fetal cells were found only in those cases in which the cord serum as well as the maternal serum contained demonstrable Rh antibodies.

The whole problem of demonstration of antibodies in mother and child as well as attempts to correlate antibody titers with severity of erythroblastosis must now be re-examined in the light of new information obtained by the use of antihuman globulin serum as a developing test for Rh antibodies. Because the test yields observable agglutination it seems to offer some advantage over the blocking test in the clear-cut nature of the results and in its sensitivity. It was further suggested that the test might be used to detect sensitized red cells in the infant when Rh antibody could not otherwise be shown. Since adopting this method as a routine developing test in suspected Rh isoimmunizations, we have been able to confirm its advantages in respect to its sensitivity for incomplete antibodies and to prove its usefulness in demonstrating Rh antibody in the serum of mother and infant when other tests failed.

Of even greater interest is the fact that the developing test not only detects incomplete or blocking antibodies but also detects specific Rh antibodies not demonstrable with agglutinating or blocking tests. The developing test also shows agglutination when agglutinating Rh antibodies are present, but the clumping reaction is much stronger than that of the agglutinating antibody alone. In our cases it may be noted from Table I that the developing titer equalled, or far exceeded, the agglutination or blocking titers. In Cases 5 to 8 there are instances of developing titers in mother or baby much higher than agglutinating or blocking titers and even examples of positive tests when the other two tests were negative. This evident difference between the three tests indicates that the basic character of Rh antibodies must be different. In the case of the agglutinin which saturates the hapten and produces the visible reaction of clumping, the classical antibody is seen. In the case of the blocking reaction an antibody is present which is capable of specific hapten saturation without the production of clumping. However, in the case of the immune globulin demonstrable by the developing serum, the antibody apparently is capable of specific adsorption without saturation of the available haptens and without the

production of agglutination. That this latter reaction is specific can be proved by the fact that the adsorption of this immune globulin occurs only with the Rh antigen and not with the O, A, B, M, and N antigens. The unique feature of this developing reaction in not saturating the available haptens is further demonstrated by the fact that after Rh-positive cells are subjected to the immune globulin no interference is found with the agglutinability of these cells with known Rh-typing serum. For the sake of brevity and in line with the designation of agglutinating and blocking Rh antibodies, we have designated this third type as developing antibodies. It seems possible that these developing antibodies may represent a third order in respect to valence as compared to blocking and agglutinating antibodies, arising as incomplete or fractional units of the other two forms.

This developing antibody is of great clinical importance, since it has been observed by many investigators that clinical erythroblastosis may occur in the Rh-positive child of an Rh-negative mother in whom no antibodies were evident. Likewise, it has been noted that the observed titers of maternal antibodies have shown little or no correlation with the severity of the disease in the infant. Levine²³ wisely attributed this inconsistency to the limitations in the sensitivity of the techniques then available. The demonstration of the blocking effect by Race¹⁷ and Wiener¹⁸ gave the first evidence that we were dealing with immune substances previously hidden to the ordinary agglutinating test. When the developing test was applied to our cases (Table I), further evidence of hidden antibody titers became apparent. In Case 8, for example, the agglutinating titer of 1:2 and blocking titer of 1:32 certainly did not suggest the severe form of erythroblastosis represented by the macerated hydropic fetus. However, the developing titer of maternal antibodies of 1:2048 correlates well with the severity of the injury to the infant. In Cases 6 and 7 relatively mild clinical courses could be expected from the moderate developing titers. From the older Cases 1 to 5 conclusions must be drawn with caution because it may be assumed that considerable loss of titer may have occurred during the time the serum was preserved in the frozen state, although even here the developing titer was equal to, or greater than, the original agglutinating titer. The correlation of titer and severity of erythroblastosis must not be overstressed because the individual resistance of each baby involving questions of compensatory production of red cells, liver function,²⁷ and other factors is to be considered.

The application of the developing test to the erythrocytes of the newborn shows promise of giving us a specific test for erythroblastosis. The brief evidence to date suggests that this test may satisfy the requirements for sensitivity and specificity to fill this role. Since adoption as a routine test sensitized fetal red cells have been found in three cases, two with clinical hemolytic disease and one in subclinical form but with suggestive maternal history (Table I). In all four cases mothers were Rh negative and infants Rh positive. It should be noted that the developing test as applied to washed infant's cells is not necessarily specific for Rh antibodies but presumably would detect any specifically adsorbed antibody, for example, anti-Hr on Rh-negative cells or anti-A on A erythrocytes.

The developing test would also help avoid errors in instances such as Case 3 where blocking antibody adsorbed *in vivo* was sufficient to make the infant appear Rh negative. In such a case, of course, the developing test would be positive. Case 6 not only illustrates the diagnostic value of the developing test but also shows evidence of continuing adsorption of antibodies through the mother's milk. In this instance a strictly laboratory diagnosis of erythroblastosis was made on the basis of a 4 plus reaction of washed infant's cells to the antihuman globulin serum. The deterioration in the child's condition after discharge from the hospital and the continued strong developing test on the baby's cells can be attributed to the breast feeding. After stopping breast feeding the adsorbed antibodies were present nine days later, but seventeen days thereafter no sensitized infant erythrocytes could be found.

The specificity of the developing test is supported by the negative reactions obtained in stillborn infants and cases of jaundice of the newborn in which no evidence of erythroblastosis could be found. In most of these the Rh type was the same as the mother. If this specificity can be completely established in routine use, this test appears to be of the utmost practical value for diagnosis, prognosis, and treatment.

Analysis of the reported cases of hemolytic disease of the newborn brings out two therapeutic considerations. First, the use of early and adequate transfusion of Rh-negative blood, utilizing the cord route in severe cases, is urgently recommended. In Cases 3 and 9, with symptoms of kernicterus, transfusion was delayed three and two days, respectively. However, in Case 1, with a previous child suffering from kernicterus, a high maternal antibody titer, and strongly positive fetal eluate, the infant recovered completely. In this case a transfusion of 100 c.c. was given in the first hour after birth. Second, the infant should not be put on the breast if any Rh antibodies can be demonstrated, even if no clinical erythroblastosis is apparent. The developing test on infant erythrocytes probably will be sufficiently sensitive to determine this question for Rh-positive babies of Rh-negative mothers.

In addition to the use of developing serum in erythroblastosis, many other applications have been made in this laboratory. The results of the use of this method to bring out hidden titers in heterophile antibody tests and the Huddleson test for brucellosis will be reported elsewhere.

In transfusion reactions the developing serum has been useful in determining whether a specific isoimmunization has occurred either to Rh or other unidentified antigens such as those reported by Race and associates.²⁶

SUMMARY AND CONCLUSION

1. Additional evidence is presented which tends to establish certain steps in the pathogenesis of erythroblastosis.

2. Three varieties of Rh antibodies, namely, agglutinating blocking, and developing, found in the newborn as well as the mother are described.

3. Evidence is presented to show that the Rh antibody acts as a hemolysin *in vitro*.

4. Analysis of ten cases of erythroblastosis shows relatively good correlation between severity of the disease and antibody titer as demonstrated by the developing test.

5. Results of therapy in these cases suggests the advisability of immediate and adequate transfusion of Rh-negative blood in hemolytic disease of the newborn.

6. Breast feeding is contraindicated when Rh antibodies can be demonstrated in the maternal or fetal serum or adsorbed on fetal erythrocytes.

7. Evidence is presented indicating a high degree of sensitivity and specificity for the developing test as used herein to demonstrate isoantibodies against human erythrocytes.

8. The developing test as applied to the demonstration of adsorbed antibodies on fetal erythrocytes is suggested as a diagnostic test for erythroblastosis.

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BLOOD TRANSFUSION STUDIES

I. THE DIFFERENTIATION OF HEMOLYTIC AND NONHEMOLYTIC TRANSFUSION REACTIONS

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IN THE past the classification of transfusion reactions has depended mainly on the degree of fever and the severity of the clinical manifestations, but there has not been any general acceptance of a classification which differentiates reactions associated with hemolysis from those without hemolysis. The importance of examining the plasma for hemoglobinemia after every febrile transfusion reaction has been emphasized by various authors,¹⁻⁷ and the recognition of its occurrence and fundamental importance has been well known for years. Nevertheless, in many reports of fatal transfusion reactions information regarding hemoglobinuria is lacking, and in most reports no mention is made of hemoglobinemia.

The incidence of reactions of all kinds varies with different reports but usually ranges from 3 to 5 per cent. Erf and Jones⁸ report an incidence of 3.2 per cent in a series of 2,889 transfusions. In this series only two transfusions resulting in nonfatal reactions were found to be due to incompatible blood, and urticaria developed in 0.3 per cent of the patients. In 1942 Levine⁹ reported the incidence of reactions as 4.2 per cent of 522 consecutive blood transfusions and 2.0 per cent of 149 consecutive plasma infusions. Of these none were hemolytic. Wiener and co-workers³ described two cases of incompatibility in 3,000 transfusions. Carlson¹ reported that 6.0 per cent of 3,388 transfusions of banked blood resulted in reactions. Of these eleven cases, or 0.32 per cent, were serious. These included three hemolytic reactions (one death), three cases of jaundice without other evidence of hemolytic reaction, two anaphylactic reactions, and three cases in which cardiovascular embarrassment was prominent. In a series of 43,284 transfusions collected from the literature, the incidence of hemolytic reactions was 1.8 per 1,000 transfusions and the mortality 1.4 per 1,000 transfusions.¹⁰

Wiener and Shaeffer² state that blood older than ten days caused chills and fever quite regularly. Icterus could not be detected in patients receiving blood less than eight days old, while in patients receiving older blood it occurred regularly, at times together with hemoglobinemia and hemoglobinuria. Most investigators agreed that unless the blood is older than nine days the age of blood does not influence the incidence of febrile reactions. DeGowin and associates¹¹ have demonstrated that the potassium content of the plasma of stored blood

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does not cause any untoward symptoms or signs. They have warned against the heating of donor's blood because of the occurrence of hemolytic reactions attributable to overheating of blood. No febrile reactions could be attributed to administration of blood at room temperature.

Hemolytic reactions are most often due to group incompatibility or to the occurrence of isoagglutinins, such as anti-Rh agglutinins, and occasionally to overheated blood. Hesse¹² stresses the use of Group O blood which has not been cross-matched as a cause of fatal reactions. Many of these reactions may have been due to the Rh factor, just as were many severe hemolytic intragroup reactions which were unexplained before discovery of the Rh factor. Anbert and co-workers¹³ demonstrated that 40 per cent of Group O donors have anti-A titers of over 1:512; they noted that transfusions of Group O blood of such high anti-A titer, or higher, rarely produced an elevation of the red blood cell levels of Group A recipients. Severe or fatal hemolytic reactions did not occur. The use of low titer anti-A Group O plasma or blood is satisfactory. There have been many reports of the safe use of universal donors. In France the Transfusion Sanguine d'Urgence of Paris¹⁴ had reported no fatalities due to hemolytic reactions up to 1939; Group O donors formed almost the sole source of blood for the service. Hardin¹⁵ compared two series of transfusions in the Army Medical Service. There was no difference in the reaction rates between the 7,299 transfusions of group specific blood and the 9,392 transfusions of universal donor blood.

Transfusion reactions generally fall into the following groups: (1) simple febrile reactions due to pyrogenic substances in the solutions, (2) allergic reactions, both anaphylactic and urticarial, (3) circulatory reactions from too rapid administration particularly to seriously anemic and debilitated patients, and (4) febrile reactions accompanied by intravascular hemolysis. DeGowin¹⁶ has also recognized a group with jaundice but without intravascular hemolysis. Separation of hemolytic reactions from the other varieties, and especially the simple febrile reactions, is often impossible on the basis of clinical symptoms and signs. The fever and chills may be equally severe, and the patient may appear just as ill with either form. Hesse¹⁷ has stressed the importance of back pain and pain in the legs as evidence of spasm of the renal vessels immediately after a reaction to incompatible blood. It is true that many of the patients with transfusion reactions followed by renal insufficiency have varying degrees of backache and general body aches, but there are enough patients with no characteristic symptoms to make a differentiation difficult or impossible by symptomatology alone. Furthermore, aching pains may occur even after a pyrogenic reaction to plain crystalloid solutions administered intravenously. When blood is administered during anesthesia, fever alone may occur, the subjective reaction being entirely absent, and yet the patient may have a severe hemolytic reaction. When anuria occurs after an operation accompanied by transfusions, one should consider a hemolytic reaction as a likely cause and look for hemoglobinemia immediately in order to establish the diagnosis.

METHOD OF STUDY

In order to ascertain the significance of hemoglobinemia in reactions to transfusions, it was necessary to determine what would occur after ordinary transfusions. Therefore, a study was made of a series of fifty-eight transfusions given to patients with diseases associated with anemia. Blood specimens were obtained just before the transfusion was to be given and within the hour after its completion. The Malloy and Evelyn method¹⁸ was used to determine serum bilirubin concentrations in forty-two patients before the blood was given at five hours and again from eighteen to twenty-four hours afterward. The blood for plasma was collected with a clean dry syringe and needle; 4.5 c.c. of blood were allowed to run by gravity into a graduated centrifuge tube containing 0.5 c.c. of 3 per cent sodium citrate solution. The tube was inverted gently only once and the cork removed. The blood was centrifuged immediately and the plasma volume recorded. The plasma was then separated from the cells using a medicine dropper.

The technique of hemoglobin determinations¹⁹ is briefly as follows: The reagents are chemically pure pyridine, dilute ammonium hydroxide (1 per cent), from 0.2 to 0.4 per cent sodium hydrosulfite solution freshly made with dilute ammonia, and 3 per cent hydrogen peroxide. In an Evelyn colorimeter tube 1 c.c. (or less) of plasma is diluted to 7.5 c.c. with 1 per cent ammonium hydroxide, after which 0.5 c.c. of pyridine and 2.0 c.c. of fresh sodium hydrosulfite solution are added. A "blank" tube is set up simultaneously with all these ingredients, except that there is 1.0 c.c. less of ammonia to allow for 1.0 c.c. of 3 per cent hydrogen peroxide. Five minutes are allowed for decolorization of the contents of the "blank" tube. Using a 550 m μ filter, one determines the galvanometer deflection of the Evelyn colorimeter in the usual manner. The concentration is then determined as follows (appropriate correction has to be made if less than 1.0 c.c. of plasma is used):

$$\text{Concentration} = \frac{1000 \times L}{K} = \text{mg. hemoglobin per 100 c.c.}$$

¹L = (2 - logarithm of the galvanometer reading)

K = 1.843

The concentration is corrected for dilution by multiplying the value obtained by the factor: $\frac{\text{Volume of supernatant fluid}}{\text{Volume of supernatant fluid} - 0.5 \text{ c.c.}}$. Normal values with this method range from a trace to 6.0 mg. per 100 c.c. of plasma. Normal values obtained by the benzidine method of Bing and Baker²⁰ range from a trace to 5.0 mg. per 100 c.c. of plasma.

In four of fifty-eight patients with anemia the plasma hemoglobin level before transfusion was higher than 6.0 mg. per 100 cubic centimeters. Determinations on the plasma of each of these patients were repeated within an hour afterward and before the time of another transfusion and were found to be less than 6.0 mg. per 100 c.c., so it is safe to conclude that slight artificial hemolysis had occurred at the time of the collection of the first blood specimen

TABLE I. HEMOGLOBIN LEVELS BEFORE TRANSFUSION

MG. HB. PER 100 C.C.	NUMBER	PER CENT
PLASMA		
0 to 3	48	83.0
3.1 to 6	6	10.2
6.1 to 9	2	3.4
9.1 to 12	2	3.4
Total	58	100.0

(see Table I for the distribution of values for plasma hemoglobin before transfusion). It is important to note that hemoglobin is not visible in plasma until the level is higher than 20 to 25 mg. per 100 c.c.; therefore, hemoglobin is not visible in properly obtained normal plasma or serum but is easily visible when any serious intravascular hemolysis has occurred recently.

In Table II is shown the range of hemoglobin concentrations in specimens of plasma obtained within the first hour after transfusion. Old blood is used to designate bank blood older than three days. There are a few values above 6.0 mg. but none higher than 12.0 mg. per 100 cubic centimeters. There was an average increase over the pretransfusion values of about 1.0 mg. per 100 cubic centimeters. This can be accounted for in part by the fact that a small amount of hemoglobin was found in the transfused plasma. It is safe to say that no intravascular hemolysis occurred in any case.

Transfusions With Febrile Nonhemolytic Reactions.—Nineteen febrile reactions to transfusions were studied, which proved to be nonhemolytic (see Table II). Nine patients had a temperature elevation to 103° F. or higher, and all had chills and some fever. Specimens were obtained within an hour in each instance, and in some instances later specimens were also obtained. The distribution of values is practically the same as those obtained after uncomplicated transfusions. It was gratifying to know that no hemolysis had occurred, even though some of the reactions were very severe from the standpoint of clinical symptoms.

TABLE II. PLASMA HEMOGLOBIN LEVELS AFTER EIGHTY-TWO TRANSFUSIONS

MG. HB. PER 100 C.C. PLASMA	GROUP WITH NO REACTIONS						GROUP WITH FEBRILE REACTIONS	
	OLD BLOOD		FRESH BLOOD		TOTAL			
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
0 to 3	15	62.5	24	61.5	39	62.0	7	36.8
3.1 to 6	4	16.7	9	23.0	13	20.6	9	47.4
6.1 to 9	3	12.5	5	12.8	8	12.7	2	10.5
9.1 to 12	2	8.3	1	2.7	3	4.7	1	5.3
Total	24	100.0	39	100.0	63	100.0	19	100.0

Transfusions With Hemolytic Reactions.—In Table III are summarized some of the pertinent data in five cases of hemolytic reactions to transfusion studied during the years 1942 to 1944. Patients L. H. and M. K. did not develop hemoglobinuria. Patient L. H. was given 200 c.c. of blood before she had a severe chill, pain in the back, and a fever of 104° Fahrenheit. Patient M. K. was given 500 c.c. of blood before she had a severe chill and a fever of 103.6° Fahrenheit. It is possible that Patient M. K. had a higher level than 124 mg. per 100 c.c., because three hours had elapsed after the reaction. At any rate the threshold

TABLE III. HEMOLYTIC REACTIONS TO TRANSFUSION

PATIENT	TIME AFTER TRANSFUSION (HR.)	PLASMA HEMOGLOBIN (MG./100 C.C.)	METHEM- ALBUMIN	OLIGURIA	HEMO- GLOBINURIA	BILIRUBIN (MG./100 C.C.)
L. H.	½	36				
	2	38				
	12			0	0	2.5
M. K.	3	124				
	5	75				3.3
	18			0	0	2.7
J. N.	24	54			+	
	30	43	0	0	0	
	40	12			0	4.6
M. B.	42	38	0	0	+	
	48	33			0	6.1
	67	16			0	
	96					.47
J. L.	20	254	0	+	+	
	44	160	+	+	+	Jaundice

for hemoglobin was not exceeded in either instance, and the true nature of the reaction would have been unrecognized if hemoglobinemia had not been found. Neither patient was jaundiced beforehand, but, as indicated in Table III, transient elevation of bilirubin values afterward substantiated the occurrence of a hemolytic episode. Visible jaundice was very slight or questionable.

The last three patients received transfusions during major operations, so that they did not manifest the usual clinical evidence of reaction.

Patient J. N. was given 500 c.c. of Group O blood, 540 c.c. of Group O plasma and 250 c.c. of Group A plasma during an operation for the ligation of a patent ductus arteriosus. It may be noted that the first plasma specimen was obtained twenty-four hours afterward and that hemoglobinuria was present up to this time but absent thereafter.

Patient M. B. was given 2,000 c.c. of blood during a lobectomy for bronchial adenoma. Hemoglobinuria continued until the forty-second hour but was absent thereafter. In patients J. N. and M. B., hemoglobinuria was responsible for the recognition of hemolysis but this was a long time after the transfusions in both instances. The serum bilirubin concentrations were increased in the first four patients after the transfusion reactions but quickly returned to normal. The bilirubin was indirect reacting in each instance so only the total bilirubin is recorded. Recovery was uneventful in each of the first four instances. No signs of renal damage occurred. Neither Patient J. N. nor Patient M. B. had methemalbumin²¹ in the plasma on ordinary spectroscopic examination, but Schumm's test for hemochromogen was not carried out.

Patient J. L. was given multiple transfusions during a craniotomy. Oliguria and hemoglobinuria were noted after operation. Severe oliguria continued until the patient's death seventy-six hours later; only 25 to 35 c.c. of urine were obtained by catheter during each twenty-four hour period. The blood urea nitrogen was 103 mg. per cent just before death. Jaundice was present, but the quantity of bilirubin was not determined. Methemalbumin²¹ with an absorption band at 623 to 624 mμ was recognized in the plasma specimens at forty-

four hours and sixty-eight hours after the first transfusion but was not present at twenty-four hours. The addition of sodium cyanide to the plasma did not abolish the characteristic band nor change its intensity visibly, so that it could not have been due to methemoglobin. Unfortunately, permission for autopsy could not be obtained in this case.

It is not possible to estimate the initial plasma hemoglobin levels in these last three instances; however, since hemoglobin is cleared very rapidly from the blood stream, it may be assumed that the initial levels must have been much higher in each case. Obviously Patient J. L. suffered the most severe reaction as evidenced by the plasma hemoglobin levels and the fatal renal injury with urinary suppression. Gilligan and associates²² injected 16 Gm. of hemoglobin into an adult, and the plasma was cleared of hemoglobin in twelve hours. If all the cells in 500 c.c. of blood were hemolyzed, from 60 to 75 Gm. of hemoglobin would be liberated giving plasma levels of 2,000 to 2,500 mg. per 100 c.c. (assuming a plasma volume of 3 liters).

DISCUSSION

The absence of hemoglobinemia following transfusions which do not result in any kind of reaction has been demonstrated. The absence of hemoglobinemia after certain febrile transfusion reactions is important evidence in separating such reactions from hemolytic reactions. Two patients with hemolytic reactions and early, transient hemoglobinemia and without hemoglobinuria substantiate the contention that hemoglobinemia is a fundamental and pathognomonic feature of intravascular hemolysis. One patient developed anuria and died as a result of uremia. This patient had the highest plasma hemoglobin levels observed in any of the five patients with hemolytic reactions; this observation lends support to the thesis that the plasma hemoglobin level is an important factor in the development of renal damage. In each of the last three cases cited observations were made a long time after the transfusions, so one cannot compare the findings with any observations except those made at the same time interval, since hemoglobin disappears from the blood stream at a rapid rate.

Although infrequent, hemolytic transfusion reactions are the chief hazard of blood transfusions. It is important to recognize the occurrence of hemoglobinemia whenever intravascular hemolysis has occurred and to obtain and examine the serum or plasma immediately after any reaction to transfusion. To insure against artificial hemolysis, one can use isotonic sodium citrate as described under Method of Study. It would be very instructive to make serial quantitative plasma hemoglobin determinations whenever intravascular hemolysis has been sufficiently great to color the plasma. The quantity of hemoglobin excreted in the urine should be determined at appropriate intervals depending on the individual case.

Whenever a febrile nonhemolytic reaction to transfusion has occurred, every effort should be made to determine its cause. The contents of the transfusion set should be examined for bacterial contamination. The tests for compatibility of donor and recipient should be repeated carefully. The patient should be observed for the development of signs of shock, and the fluid intake and output should be recorded carefully.

CONCLUSIONS

Hemoglobinemia does not result from transfusion of fresh compatible blood. The importance of distinguishing hemolytic from nonhemolytic transfusion reactions has been emphasized. Hemolytic reactions are characterized by hemoglobinemia, whereas simple febrile reactions are not. There is a need for more quantitative observations in patients with hemolytic transfusion reactions made immediately after the reaction and followed serially until hemoglobinemia disappears. All febrile reactions should be studied early and every effort made to determine the etiology of the reaction.

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BLOOD TRANSFUSION STUDIES

II. THE SURVIVAL OF FRESH AND STORED TRANSFUSED ERYTHROCYTES AS DETERMINED BY UROBILINOGEN EXCRETION

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NUMEROUS studies have been devoted to the fate of the transfused erythrocyte, the effect of storage on its *in vitro* properties, and the effectiveness of various preservative solutions. The most important criterion for assessing the value of the transfused erythrocyte is its *in vivo* survival time. The fate of the transfused erythrocyte has been studied by three methods: the differential agglutination method of Ashby¹ and Wiener²; the use of radioactive iron which is incorporated in the hemoglobin molecule³; and the study of urobilinogen excretion following transfusion.⁴

In Ashby's agglutination method a Group A or Group B recipient is transfused with Group O blood. Periodically thereafter small samples of blood are taken. The recipient's cells are selectively agglutinated with Group O serum leaving the donor's cells free to be counted. Wiener's method² is similar in principle but makes use of the M and N subtypes of blood.

Recently Ross and Chapin⁵ have utilized the radioactive isotope of iron for the identification of transfused blood cells. When fed or injected into persons with hypochromic anemia of simple iron deficiency, this isotope is incorporated into the hemoglobin of newly formed erythrocytes. Blood was drawn from these donors into flasks containing 2.5 per cent sodium citrate solution with a pH of 7.4 and stored for periods varying from one to fourteen days at 10° C. in a dark refrigerator. From 40 to 50 c.c. of this labeled blood were injected intravenously into healthy adults all of whom had normal hemoglobin levels. At varying intervals of time after the injection of blood, samples were withdrawn for blood volume determinations and for counts of radioactive cells by the use of the Geiger-Müller apparatus.

The study of urobilinogen excretion before and after transfusion was used in the present study and will be discussed in more detail later.

It is generally agreed by most investigators^{1-3, 5, 6, 10, 12} that the average *in vivo* survival time of fresh transfused erythrocytes is more than thirty days and that some survive as long as 120 days. Fresh blood is composed of cells of varying ages, and their potential life is inversely proportional to their age, even under the most favorable circumstances. Data collected by Mollison and Young^{6, 7} show that about 85 per cent of fresh transfused blood cells are still present at the end of two weeks, 70 per cent at the end of four weeks, and 45 per cent at the end of eight weeks.

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As the storage time of citrated blood increases the red cell survival time decreases. The data of various investigators on the survival time of stored citrated blood of varying ages are not in complete agreement and do not lend themselves very well for comparison. However, from the data available, it appears that citrated blood stored three days or less is equal to fresh blood in respect to survival. According to Mollison and Young,⁷ citrated blood five to nine days of age has a fairly good survival; that is about 50 per cent of the cells were still viable at three weeks and 27 per cent at the end of two months. On the other hand, Belk and Barnes⁸ state that blood stored seven days or more in sodium citrate solution survived no more than forty-eight hours. Citrated blood stored between four and six days occupies an intermediate position and probably should be considered only moderately effective in respect to survival time.

Ross and Chapin³ studied the twenty-four hour survival of erythrocytes by means of radioactive iron and found that only 60 per cent of six-day-old cells and about 5 per cent of ten-day-old or older cells survived for twenty-four hours, that is, were found in the circulating blood.

As early as 1916 Rous and Turner¹¹ found that the addition of glucose to the citrated blood of experimental animals enhanced the keeping qualities and the *in vivo* survival of transfused blood. However, the large volume of citrate and glucose solution used by them made it impractical. Since then glucose-citrate solutions of smaller volume have been used with excellent results in regard to convenience and survival of the transfused erythrocytes.⁹ When these mixtures are used the cell survival time depends somewhat upon the concentration of glucose and citrate, but the optimal range is quite wide. It has been found that variation of the concentration of glucose in the final mixture from 0.6 to 2.2 per cent makes little difference to subsequent survival. A citric acid-sodium citrate-glucose mixture can be autoclaved without caramelization of the glucose, but a sodium citrate-glucose mixture will be caramelized by autoclaving. Immediate storage of donors' blood at a temperature between 4 and 10° C. and avoidance of actual freezing are essential.

Dacie and Mollison¹² showed that blood taken from a patient with familial hemolytic anemia both before and after splenectomy disappeared rapidly when transfused into a normal recipient. On the other hand, normal blood transfused into six patients with familial hemolytic anemia survived normally in five, and in one patient cell survival was somewhat diminished.

PIGMENT STUDIES FOLLOWING FRESH AND OLD BLOOD TRANSFUSIONS

Strumia¹³ and Wiener and Schaeffer¹⁴ have noted hemoglobinemia following transfusion of citrated blood older than ten days. Transient bilirubinemia has also been observed following the use of blood stored for more than five days compared to that stored for shorter periods.¹⁴ Wasserman and associates⁴ in a similar study on three patients with hyporegenerative anemia found an increase in feces urobilinogen excretion roughly proportional to the time of storage of citrated blood. Fresh blood usually caused no, or only a very slight, increase

in urobilinogen excretion. In their last case⁴ blood stored in glucose-citrate mixtures caused less rise in urobilinogen than when stored in citrate alone.

In order to study the relative efficiency of the blood in the University Hospital blood bank, the values of the serum bilirubin, plasma hemoglobin, and feces urobilinogen were observed before and after fresh and old blood transfusions. The donor blood was drawn into sterile flasks containing 50 c.c. of 2.5 per cent sodium citrate and stored at 4 to 10° Centigrade. Although not as accurate quantitatively as the actual counting of surviving donor cells, the urobilinogen excretion roughly indicates the rate of cellular destruction after transfusion. After the preliminary studies, fresh blood (stored less than twenty-four hours) was given over a period of one to one and one-half hours. Plasma hemoglobin levels and serum bilirubin were determined within an hour and again five hours after transfusion. Quantitative feces urobilinogen studies were carried out for the next eight days. After an interval of eight days or longer, the same studies were repeated following transfusion of old blood (stored from six to eight days). The amount of blood was 450 c.c., except in one case where two transfusions were given in succession instead of one. The urobilinogen was determined in each instance by the method of Watson,¹⁵ the bilirubin by the method of Malloy and Evelyn,¹⁶ and the plasma hemoglobin by the method of Flink and Watson.¹⁷

The first four patients had severe hyporegenerative or aplastic anemia. Patient H. had diabetes mellitus and chronic osteomyelitis, and Patient C. T. had chronic glomerulonephritis with uremia. None of the patients experienced untoward symptoms following these transfusions.

RESULTS

The plasma hemoglobin showed no appreciable change following any of the transfusions. No significant intravascular hemolysis took place following either fresh or old blood transfusions (Table I).

TABLE I

PATIENT	INITIAL HEMOGLOBIN	MAXIMUM PLASMA HEMOGLOBIN (MG./100 C.C.)		INCREASE IN BILIRUBIN CONCENTRATION (MG./100 C.C.)	
	(GM./100 C.C.)	BEFORE	AFTER	FRESH BLOOD	OLD BLOOD
B. D.	5.84	7.0	1.4	.39	2.55
L. M.	2.2	1.5	3.4	.32*	2.8
C. A.	4.35	1.0	4.2	.0	1.68
O. H.	4.15	1.1	9.0	.35*	.51*
H.	8.6	2.7	6.8	.20	.84
C. T.	4.4	1.3	3.9	.18	.45

*Average of two transfusions on successive days.

In all cases studied there was a greater rise in the bilirubin concentration after the old blood than after fresh blood. The maximum bilirubin concentration was found usually at five hours after transfusion. This finding is in agreement with the observations of others.^{13, 14}

TABLE II. UROBILINOGEN EXCRETION IN FECES (MG. PER DAY)

PATIENT	FRESH BLOOD TRANSFUSIONS			OLD BLOOD TRANSFUSIONS		
	BEFORE	AFTER	INCREASE	BEFORE	AFTER	INCREASE
B. D.	138	103	-	139	234	95
L. M.*	45	65†	20	131	268	137
C. A.	71	73	2	72	139	67
O. H.‡	157	98†	-	63	134†	72
H.	55	26	-	42	163	121
C. T.	47	128	81	88	145	57
Average	85	82		89	181	92

*Transfusions on two successive days, fresh blood.

†Result after two transfusions on successive days, the four-day period beginning with the first transfusion.

‡Transfusions on two successive days for both the fresh and old blood studies.

Feces urobilinogen excretion (Table II) was considerably increased after old blood but not after fresh blood in Patients B. D., L. M., C. A., and H. Patient O.H. excreted more urobilinogen after old blood than in the control period but excreted less than the control value obtained before the first transfusion. In Patient C. T. the increase in urobilinogen was slightly greater after fresh blood than after old blood. In five out of six patients studied it was apparent that an increased rate of destruction of blood must have taken place after the use of old blood compared with that following fresh blood.

CONCLUSIONS

It is evident that there is an increased extravascular destruction of red blood cells after transfusion of old blood, for there is a significant rise in the serum bilirubin and feces urobilinogen excretion after such a transfusion. Blood stored in sodium citrate for more than six days is less efficient than fresher blood in the therapy of anemias of any kind. This is supported by the pigment studies previously described and also by the studies of others, based on differential agglutination and the use of radioactive iron.

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BLOOD AND BONE MARROW IN INFECTIOUS MONONUCLEOSIS*

A REVIEW OF THE LITERATURE AND A REPORT OF TWENTY-FIVE CASES

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SINCE the original descriptions of infectious mononucleosis by Pfeiffer¹ (1889) and others before the turn of the century, an enormous literature has accumulated pertaining to the clinical aspects and laboratory characteristics of the disease. The introduction by Paul and Bunnell² (1932) of the serologic test whereby suspected cases of infectious mononucleosis could be diagnosed without question served to stimulate new interest in the disease.

Although numerous articles in the literature touch on the hematologic findings in infectious mononucleosis, none of them have added much to Downey's³ classical morphologic description of the blood. His original colored plate with the description of the cells seen in this disease compared with those seen in cases of leucemia has never been surpassed.

Studies of the bone marrow in cases of infectious mononucleosis have been surprisingly few in number. It is only in the last ten years that any attempt has been made to correlate the blood findings with the cellular pattern in the bone marrow. It is of interest to note, in view of the fact that the disease may be mistaken for leucemia, that the first examinations of the bone marrow in this disease were made by Freeman⁴ (1936) who suggested that infectious mononucleosis may really be an abortive, benign form of acute lymphatic leucemia. It is surprising that no one has challenged this investigator's conception of the malignant potentiality of the atypical lymphocytes seen in infectious mononucleosis. Read and Helwig⁵ analyzed 300 cases of infectious mononucleosis and without examining the bone marrow arrived at the conclusion that the cause of the anemia, leucopenia, and thrombopenia in some of their cases was due to depression of the activity of the bone marrow resulting from an infectious granulomatous process involving the marrow.

REVIEW OF THE LITERATURE ON THE BONE MARROW IN INFECTIOUS MONONUCLEOSIS

Freeman⁴ was the first to describe the bone marrow in cases of infectious mononucleosis. He reported two cases, one in a 19-year-old student nurse with a leucocyte count of 10,400 per cubic millimeter and 76 per cent lymphocytes and a second case in a 55-year-old white woman with a white blood count of 23,600 per cubic millimeter and 91 per cent lymphocytes. The leucocyte count

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in the second case rapidly increased to 392,000 per cubic millimeter, and the woman died on the seventeenth day of illness. The heterophile antibody test was not performed in either of the two cases. The bone marrow of the sternum in both cases was filled with both immature and mature lymphocytes. Histologic sections of the bone marrow showed a lymphocytic infiltration. Freeman suggested that glandular fever may really be an abortive, benign form of acute lymphatic leucemia.

Young and Osgood⁶ stated that in infectious mononucleosis the typical large lymphocytes were demonstrated to be present in the sternal marrow as well as in the blood. There is no mention as to the number of cases they studied.

Nordensohn⁷ examined the sternal marrow in thirteen cases of infectious mononucleosis and observed a toxic alteration of the myeloid cells with a moderate shift to the left of the granulocytic series. Erythropoiesis was normal. There were no morphologic indications of a lymphoid metaplasia in the bone marrow of the sternum.

Markoff⁸ reported on the bone marrow findings in one case of infectious mononucleosis. He observed a lymphatic and plasma cellular reaction of the marrow with an increase in the number of metamyelocytes and a decrease in all the other granulopoietic elements. Erythropoiesis and megakaryopoiesis appeared normal. According to this investigator, in spite of the increased numbers of lymphoid cells in the sternal marrow in cases of infectious mononucleosis, the differential diagnosis from the bone marrow in lymphatic leucemia is relatively simple, and there is no reason for confusing the two diseases.

Rohr⁹ who studied twelve cases of infectious mononucleosis emphasized that great care should be exercised in interpreting the cellular marrow pattern in this disease because of the variations in the marrow pattern which are directly related to the amount of admixture with peripheral blood. The bone marrow in his cases with the disease revealed a moderate amount of myeloid immaturity. He never observed a lymphoid infiltration of the sternal marrow in his cases of infectious mononucleosis.

Mallarmé¹⁰ believes that sternal puncture aids in the diagnosis of infectious mononucleosis. The bone marrow in Mallarmé's case showed a myeloid reaction with an increase in the number of mononuclear cells, large lymphocytes, monocytes, and plasma cells. Granulopoiesis appeared normal.

Weil and Perlés¹¹ observed a hypoplastic bone marrow which consisted of myeloerythroblastic tissue and mononuclear cells similar to those seen in the peripheral blood and spleen.

Schulten,¹² Klima,¹³ and Henning¹⁴ have observed varying degrees of myeloid immaturity and confirmed Rohr's⁹ observation of the lack of any evidence of a lymphoid infiltration of the bone marrow in cases of infectious mononucleosis.

Henning and Keilhack¹⁵ observed a case of infectious mononucleosis with anemia, thrombocytopenia, and a leucocyte count of 30,000 per cubic millimeter with 80 per cent lymphocytes. The sternal aspiration was rich in cells of the granulocytic series with a few elements belonging to the mononuclear series.

Ustvedt¹⁶ studied the blood and bone marrow in one case of infectious mononucleosis. This 29-year-old man had a leucocyte count that varied between 7,000 and 10,000 per cubic millimeter and the differential count showed between 60 and 89 per cent atypical lymphoid cells. The bone marrow was hypocellular and consisted of granulocytes in various stages of development, a few erythroid cells, and lymphoid cells. The uniform type of "blast" cells seen in the marrow in cases of acute leucemia were absent. Ustvedt observed groups of mononuclear cells of a lymphoid type in the bone marrow of his case of infectious mononucleosis which were quite different from those seen in the peripheral blood and concluded that the question of lymphoid metaplasia in the bone marrow could not be eliminated. He suggested sternal puncture as an aid in ruling out leucemia.

Fieschi¹⁷ examined the bone marrow in a case of infectious mononucleosis and found a cellular marrow with a decrease in the number of mature neutrophils and a few atypical granuloblasts similar to those seen in cases of acute infection. Lymphocytes were not observed, and plasma cells were few in number. The monocytoïd cells seen in the marrow were morphologically different from the lymphoid cells found in the peripheral blood. The marrow mononuclear cells presented marked immaturity which suggested their origin from reticular cells or from large atypical hemocytoïd elements. The bone marrow in no way simulated that seen in leucemia.

Scott¹⁸ observed a normal cellular pattern in a case of infectious mononucleosis. He believes the method of sternal puncture has little value in diagnosing this condition, for even the findings of a normal marrow do not exclude lymphoid leucosis.

Vogel, Erf, and Rosenthal¹⁹ examined the sternal marrow in six cases of infectious mononucleosis and found an increased number of lymphocytes. Many of the lymphocytes were of the lymphoid type. In one case Türk irritation cells were seen in the blood but were absent in the marrow, while in a second case Türk cells were present in both the blood and bone marrow. In a second article, Vogel and Bassen²⁰ reported the marrow findings in four cases of infectious mononucleosis. They found a normal number of lymphoid elements in the sternal marrow. These investigators believe that the absence of an increased number of lymphocytes in the bone marrow is of great value in ruling out the possibility of leucemia, especially when the heterophile antibody reaction is negative.

Morrison and Samwieh²¹ studied the marrow in eight cases of infectious mononucleosis and noted a left shift in the white blood series with increased red cell activity. There were many lymphocytes in the marrow, but they were never in the proportion as seen in lymphatic leucemia. The erythroid series revealed a defective maturation which the investigators ascribed to a lack of anti-anemia principle or to some disturbance in its utilization or absorption.

Mendell, Meranze, and Meranze²² reported the bone marrow findings in eleven cases of infectious mononucleosis and observed a normal leucopoiesis and erythropoiesis. The total marrow cell count ranged from 20,000 to 170,000 per cubic millimeter, and the differential count showed 1 per cent myeloblasts, 2 per cent promyelocytes, 17 per cent myelocytes, 25 per cent metamyelocytes, 36 per

cent neutrophiles, 13.7 per cent lymphocytes (normal according to these investigators is 13.5 per cent), 3 per cent monocytes, 2 per cent eosinophiles, and 0.3 per cent reticulum cells. The erythroid series consisted of 1 megaloblast, 5 erythroblasts, and 12 normoblasts per 100 white cells. According to these authors the finding of a normal bone marrow in infectious mononucleosis, despite the large number of lymphoid cells in the peripheral blood, is sufficient to eliminate the possibility of leucemia.

Piney²³ from his studies of the bone marrow in infectious mononucleosis (number of cases studied is not stated) did not find a hyperplasia of the marrow as a whole but rather a slightly hypoplastic marrow. There was no resemblance to a leucemic marrow. The cellular marrow pattern as a whole was not greatly disturbed, but there were plasma cells and monocytoïds, the latter of which were less like normal monocytes than were the similar elements that are found in the peripheral blood in this disease. The cytoplasm of the monocytoïd cells varied from a marked basophilic to that which had no affinity for dyes, and the nuclear characteristics varied from the most immature, with nucleoli, to the most bizarre and extraordinary convolution. Piney believes that there should not be any confusion between glandular fever and German measles, although in both diseases Türk and plasma cells are found in the blood. In German measles there is a great excess of plasma cells in the lymph nodes, whereas the sternal marrow contains few plasma cells. On the other hand, in glandular fever (infectious mononucleosis) the reverse is the case; the sternal marrow contains large numbers of plasma cells, while the lymph nodes contain many monocytoïd elements but a few plasma cells.

Moeschlin²⁴ simultaneously examined the sternal bone marrow, lymph nodes, and spleen in three cases of infectious mononucleosis. He observed a moderate myeloid immaturity of the marrow. The lymphoid cells in the bone marrow varied between 12 and 17 per cent, and there was a plasma cellular reaction moderate in degree. Moeschlin believes that the lymphocytes are more probably derived from blood aspirated during the puncture than from the marrow itself. In the lymph node he observed lymphoid and monocytoïd elements in mitosis as well as normal lymphocytes. The plasma cells observed in the lymph nodes in cases of infectious mononucleosis are considered to be different from those seen in the lymph nodes in rubella. In rubella, puncture of the lymph nodes reveals numerous large primitive cells with reticular nuclei containing nucleoli. These plasmoblasts were observed in various stages of maturity or transition to the typical plasma cell seen in the peripheral blood. In the lymph nodes the highest number of plasmoblasts were found on the second day of the illness, while in the blood they were found at their greatest peak on the fifth day. In the bone marrow neither the plasmoblasts nor plasma cells were found in any significant numbers. In one case, perhaps by chance, a lymph follicle in the bone marrow was punctured, and an increased number of plasma cells and plasmoblasts was observed. Moeschlin²⁵ believes that the lymph node and bone marrow plasma cells are totally distinct both in structure and origin.

Leitner²⁶ studied the sternal marrow in two cases of infectious mononucleosis and observed a marked myelocytic reaction. The lymphocytes averaged

5.6 per cent of the bone marrow differential count. He attributed the maturation arrest of the myeloid tissue in the bone marrow to hyperfunction of the spleen, and this action on the marrow results in a leucopenia and granulocytopenia in the peripheral blood.

Propp and Schwind²⁷ performed sternal punctures on two cases of infectious mononucleosis and reported on the findings in one of the cases. The marrow revealed a normal degree of cellularity and a normal distribution of cell types. There was a marked lymphocytosis, but no lymphoblasts were seen. The heterophile antibody test was negative. Propp and Schwind believe that because of the high percentage of lymphocytes in the peripheral blood in this disease a relatively high lymphocyte count will occur in the marrow, but the marrow is not being replaced by lymphocytes. They support the opinion that there are occasional small lymph nodules in normal bone marrow, and therefore an increase of lymphocytes is not necessarily of importance.

Owing to the extremely low mortality from infectious mononucleosis, there has been very little opportunity to examine the bone marrow from various sites histologically. Further, in the few cases that have come to autopsy, there has been a failure to study the bone marrow. Haken²⁸ reported on the autopsy findings in three children with supposedly infectious mononucleosis, but he neglected to examine the bone marrow. Koenigsberger,²⁹ in analyzing the cases reported by Haken, was of the opinion that they were not cases of monocytic angina but rather cases of malignant diphtheria. DuBois³⁰ reported a case of infectious mononucleosis in a 26-year-old man which was complicated by pneumonia and empyema and at autopsy revealed a hyperplasia of the reticulo-endothelial system. The bone marrow showed a practically normal erythroblastic series except for a few caryorrhetic normoblasts. The megakaryocytes, especially the immature forms, were reduced in number. The younger cells of the neutrophilic series were decreased in number at the expense of large mononuclear cells which had a cytoplasm that was basophilic and vacuolated. These cells appeared to be a part of a markedly active reticulum. There is no definite proof that this is a genuine case of infectious mononucleosis.

Ziegler³¹ reported on the autopsy findings in a 22-year-old white woman with infectious mononucleosis with a heterophile antibody test of 1:640. At one time during the course of the disease, the leucocyte count was 23,800 per cubic millimeter with 93 per cent mononuclear cells in the peripheral blood. The lesions and changes in the liver, spleen, lungs, and kidneys were described, but unfortunately the bone marrow was not examined.

MORPHOLOGY OF THE LYMPHOCYTES IN INFECTIOUS MONONUCLEOSIS (DOWNEY³)

Three types of lymphocytes, depending entirely on their morphologic features, give the blood its characteristic appearance in infectious mononucleosis. In the type I nucleus the chromatin forms a coarse network of heavy strands and masses which are not sharply separated from the parachromatin. In some of the larger cells there may be a slight tendency toward a more diffuse arrangement of the chromatin, and the lymphocytic nature of the nucleus always remains evident. The nuclei of the larger cells are frequently placed eccentrically

and are generally lobulated, indented, or kidney-shaped, and smaller cells also have irregular nuclei. The cytoplasm is quite characteristic. The degree of basophilia varies, but most of the cells are very basophilic. With Wright's stain the spongioplasm appears as dark blue or slate blue, finely granular, and of flaky material with a pale yellowish background of hyaloplasm. Its distribution

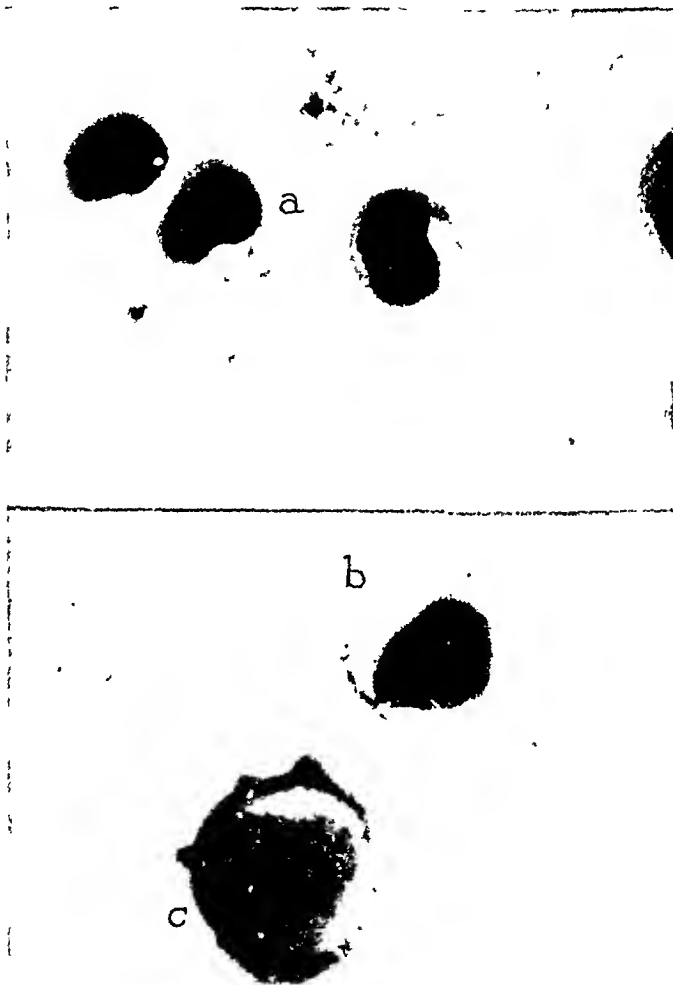


Fig. 1.—Peripheral blood films from two cases of infectious mononucleosis. *a*, Lymphocytes Downey type I. These lymphocytes were the type commonly seen in our cases. *b*, Lymphocyte Downey type III with a mature type of nucleus. *c*, Type III cell with an immature type of nucleus.

gives the cytoplasm a vacuolated, foamy, or mottled appearance. The peripheral portion may contain relatively more spongioplasm and, therefore, appear darker and more homogenous than other parts of the cytoplasm. If the nucleus is indented, the indentation is lighter than the rest of the cell because it contains relatively more of the yellowish hyaloplasm. Frequently, there are one or more

azurophilic granules embedded in the hyaloplasm of this region, which give the whole structure the appearance of a centrosphere. Most of the atypical cells contain azurophile granules, being quite abundant in most of the larger cells, and are of the lymphocytic type, that is, fine and carmine red.

In an atypical cell of type II the nucleus is somewhat similar to that of a plasma cell. The chromatin strands are very coarse, and there are several dense, rounded, or angular masses of chromatin among them. They have more of a washed appearance, and the blocking of the chromatin is not so pronounced as in the plasma cell. The cytoplasm has fewer vacuoles, and its spongionoplasm has a smoother appearance which does not give the foamy, spongy, and stippled effect noted in type I. It is generally less basophilic, and there is a more even mixing of the basophilic portion of spongionoplasm and hyaloplasm. In some cells there is the tendency of the basophilic portion of the cytoplasm to be arranged in the form of broad bands radiating from the nucleus. The cell body is generally wider than in the cells of type I, and the nucleus is rarely lobulated.

Type III cells have more leucemic features than those of types I and II. The cytoplasm is vacuolated and in some cells is quite basophilic. In the larger cells there is relatively more hyaloplasm, but the distribution of the spongionoplasm remains about the same as in type I. These cells may contain a single large vacuole with an azurophile rod and within an indentation of the nucleus in addition to smaller vacuoles distributed rather evenly throughout the cytoplasm. The nucleus may be close to that of a stem cell. There may be a diffuse, vacuolated nucleus containing nucleoli (Fig. 1).

METHODS AND MATERIAL

Twenty-five patients with infectious mononucleosis, of whom eighteen were men and seven were women, comprised the group studied. No Negroes were observed. Patients varied in age from 13 months to 35 years. Fourteen patients ranged in age from 16 to 26 years, four were less than 16 years of age, and one was over 35 years of age. Many of the patients were medical and dental students referred to the University of Illinois, and the remainder were seen in consultation because of some clinical or hematologic finding observed by the medical examiner while performing a physical examination (lymphadenopathy or splenomegaly) or by the technician while doing a differential blood count. The heterophile antibody reaction was positive in thirteen, negative in six, and the test was not performed in the remaining patients. A false-positive Wassermann reaction was not observed.

The method of sternal aspiration and the preparation of the marrow for study has been reported in detail by one of us (L. R. L.).²²

After films of the bone marrow were prepared the slide was air dried for several hours and stained with May-Grunwald-Giemsa or Wright stain and studied microscopically for cell distribution and types. When the stained films of the bone marrow were studied the ratio of myeloid to erythroid cells, the dispersion of the nucleated red cell series, the dispersion of the myeloid cell series, and the number of megakaryocytes were recorded. In recording the erythropoietic elements, the terms pronormoblast, basophilic, polychromatophilic, and

orthochromat (acidophilic) normoblast were used in order of increasing maturity. Similarly, for the granulopoietic series, myeloblast, leucoblast, promyelocyte, myelocyte, metamyelocyte, and polymorphonuclear neutrophil were used. The lymphocytes which include both normal and atypical or immature forms were recorded together and expressed as the number of lymphocytes per 100 nucleated marrow cells. Plasma cells, monocytes, and reticuloendothelial elements were noted and described as a part of the bone marrow pattern when present in greater numbers than normal, but they were not included in the counts used to determine the above percentage relationships.

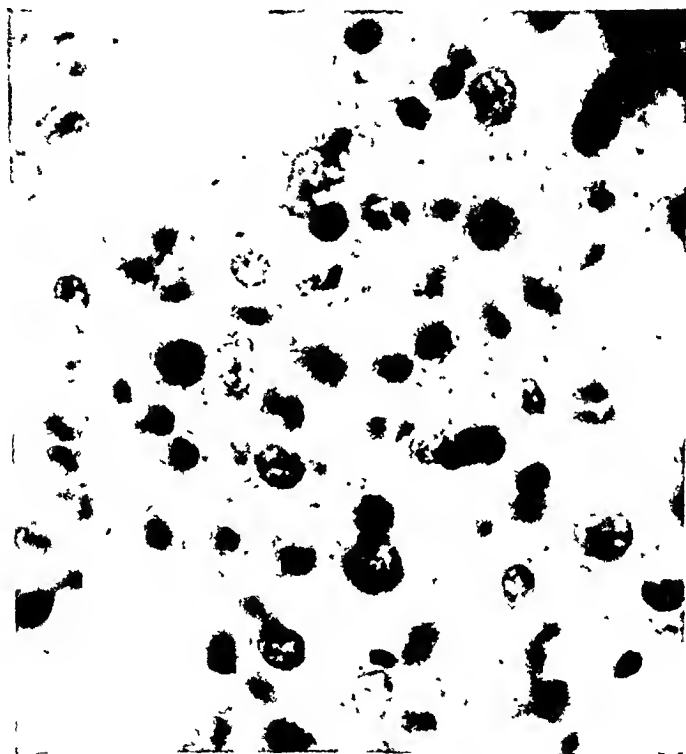


Fig. 2—Bone marrow (histologic specimen) from a case of infectious mononucleosis showing a neutrophilic myelocytic pattern with absence of lymphocytic infiltration. Many of the lymphocytes in the peripheral blood were Downey type III. Heterophilic antibody titer was 1:1224.

In a few cases of infectious mononucleosis histologic studies of the bone marrow were made from "hemopoietic particles" or "floaters" of the marrow obtained by sternal aspiration or biopsy material obtained by means of a small trephine. The marrow specimen was immediately placed in Zenker-acetic acid solution, and the sections after fixation were prepared in the usual way with paraffin and stained with eosin-azure or Giemsa. The sections were utilized in studying the topographic relationships of the marrow (Fig. 2).

Immediately preceding or following the marrow aspiration, an erythrocyte and leucocyte count, a hemoglobin determination (Newcomer's method) differen-

tial count, and a reticulocyte and platelet estimation were made with standardized and certified materials and apparatus. A hematocrit reading, sedimentation rate, and icterus index were done by the Wintrobe method.³³ Mean corpuscular volume, hemoglobin, and hemoglobin concentration were then computed.

DISCUSSION OF RESULTS AND ANALYSIS

Peripheral Blood.—Of the twenty-five patients with infectious mononucleosis, four had an anemia; in three the anemia was normocytic in type and in one, an infant 13 months of age, it was microcytic and hypochromic in character. All four patients had a positive heterophile antibody test. Except for one with a hemoglobin value of 8.5 Gm., all patients had a hemoglobin reading of 11.0 Gm. or more. In five the hemoglobin value was less than 13.5 Gm., and the highest value was 16.5 grams. The hemoglobin reading in all the patients studied ranged between 8.5 to 16.5 Gm., with an average of 13.54 grams. The mean corpuscular volume and mean corpuscular hemoglobin concentration were normal in all cases, except in the one with the microcytic and hypochromic anemia. The heterophile antibody test was positive in this instance.

The lowest erythrocyte count was 3,100,000 and the highest 6,560,000 per cubic millimeter, with an average of 4,660,000. In nine cases it was less than 4,500,000 and in only two cases was the erythrocyte value less than 4,000,000 per cubic millimeter.

The leucocyte count varied between 4,500 and 22,000 per cubic millimeter, with an average count of 10,900. In ten cases the count was less than 10,000, and in two of these cases the white count was below 5,000. In only one case was the count over 20,000. The characteristic finding in the differential count was the large number of abnormal lymphocytes which ranged from 37 to 82 per cent, with an average of 64 per cent. The atypical "leucocytoid" lymphocytes were of the type I and II of Downey³ with various degrees of variation between the types.

Extremely immature cells of the type III of Downey were rarely found in our series of cases of infectious mononucleosis. In only one case which was complicated by pulmonary tuberculosis were so-called "lymphoblasts" observed; that is, cells whose nuclear structure indicated immaturity or lacked differentiation. Some of the latter cells approached the Türk irritation form of plasma cells with diffuse myeloblastic nucleus (leptochromatic). Lymphocytic plasma cells were seen in all of the other cases as well as normal lymphocytes (mostly small types). The typical lymphoblast of acute and subacute lymphatic leucemia was not seen in our cases. Degenerated and fragile cells were absent from the blood films.

The granulocytes varied from 3 to 5 per cent. Moderate to marked toxic neutrophils with a moderate shift to the left in the neutrophilic picture were seen in all cases. The shift to the left consisted in increased numbers of non-filament granulocytes, the so-called "stab" forms, which ranged over 6 per cent in twelve cases and over 25 per cent in three cases. Neutrophilic metamyelocytes were observed in two cases, 1 per cent in the first and 2 per cent in the second

case. Myelocytes, promyelocytes, and myeloblasts were not seen. Basophilic granules or "toxic" granules, as they are often designated, were seen in the granulocytes in all the cases. The grade 3 to 4 "toxic" granulations consisted of small and large types of granules which in many instances filled the entire cytoplasm of the neutrophile. Oxidase stain on such cells revealed relatively few oxidase reacting granules (normal neutrophilic granules). Degenerative changes of the nucleus, such as condensation in different areas, were occasionally found, but fragmentation of the nucleus, swelling of the cytoplasm with vacuole formation, diffuse basophilic staining in the cytoplasm, and marked irregularity in size of the neutrophiles showing "toxic" and degenerative changes were only rarely observed.

The monocytes which were over 8 per cent in four cases were morphologically normal. The normal appearing eosinophiles were definitely increased in only one case with a value of 16 per cent. This case with a positive heterophile antibody reactive had 37 per cent atypical lymphoids and 38 stab cells in the peripheral blood at the time that the bone marrow was studied. There were only 7 per cent neutrophiles in the differential count. The basophiles were over 1.0 per cent in three cases; the highest count was 4.0 per cent in one case.

The platelets were normal in number and morphology in practically all cases and moderately increased in two cases from blood film examination.

The corrected sedimentation rate was increased in sixteen cases with values as high as 41 mm. per hour.

The reticulocyte count was normal in all cases. The icterus index was higher than 7.5 units in three cases, and in one of the cases with a value of 15 units there was a clinical jaundice.

Bone Marrow.—The bone marrow was hyperplastic in twenty cases with volumetric values as high as 35 per cent and with an average reading for all cases of 14.4 per cent. This is more than twice the normal value. The bone marrow cell counts ranged from 210,000 to 670,000 per cubic millimeter in eighteen cases, with an average count of 430,000 per cubic millimeter which is approximately one and one-half times the normal value (300,000).

The fat volume (volumetric reading) averaged 1 per cent, with the highest reading of 5 per cent in a 12-year-old boy.

The myeloid elements besides being increased in number revealed a moderate to marked degree of granulopoietic immaturity. Immaturity was never carried to the stage of myeloblastic involvement. The greatest increase occurred in the neutrophilic myelocytes which in some cases comprised over 50 per cent of the granulopoietic series. This is a marked increase when compared with the normal value of 10.8 per cent. In eighteen cases the neutrophilic myelocytes ranged over 15 per cent, and in only three cases were they less than 10 per cent. Moderate to marked degrees of "toxic" granules were observed in many of the neutrophilic myelocytes, even in phases of mitotic division (Fig. 3). The "toxic" or coarse basophilic granules which may fill the cells are different from the heavy, wine-colored or reddish lavender granulation observed in normal maturing neutrophilic myelocytes. The delicate neutral- or lilac-colored granules

which are observed in the Hof of the nucleus in many normal maturing neutrophilic myelocytes were absent, and the normal blue color seen in some of the early neutrophilic myelocytes was occasionally replaced by a diffuse basophilic staining cytoplasm. Marked degenerative changes of the nucleus or cytoplasm with the formation of the vacuoles were rarely observed. In cases of infectious mononucleosis with a predominating neutrophilic metamyelocytic type of bone marrow, the metamyelocytes were practically free of any toxic granules or degenerative changes, while the myelocytes showed the morphologic effect of a toxic process. These findings indicate that the "degenerative" shift to the left

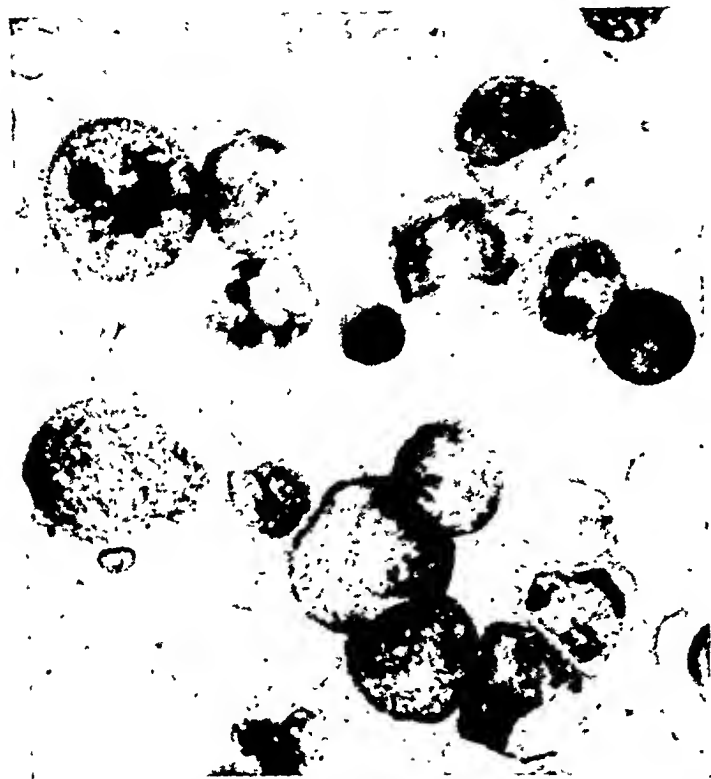


Fig. 3.—Bone marrow (concentration technique) from a case of infectious mononucleosis showing a severe toxic condition of the granulopoietic elements. Note the toxic neutrophilic myelocyte in phase of mitosis and the absence of lymphocytes in the bone marrow.

observed in the peripheral blood with an increased number of immature forms ("stab-kernige" or staff forms¹⁴), in which the nuclei are narrow, deeply staining with little or no structural detail, but more often with few or many small or large basophilic granules (toxic) in a diffuse basophilic staining cytoplasm, is the effect of toxemia on the development of the cell at the early neutrophilic myelocytic stage; that is, during the stage of specific granule formation and the beginning of nuclear configuration. In infectious mononucleosis the toxemia affects the cytoplasmic granules to a much greater extent than the nucleus, since many of the cells of the myelocytic series with toxic granules are observed in mitosis. There is no definite indication of a depressed bone marrow function

to any marked degree. The toxemia of infectious mononucleosis affects the granulopoietic tissue in the bone marrow at the early neutrophilic myelocyte stage of development to prevent the differentiation to neutrophils, so that they develop to a certain point and emerge into the peripheral blood as stab forms with varying degrees of toxic granules in the cytoplasm. Apparently those myelocytes that escape the toxic process complete their development through the several stages of neutrophilic metamyelocyte formation and become normal polymorphonuclear neutrophils (Fig. 4). Therefore, the neutrophilic metamyelocytes in the bone marrow in cases of infectious mononucleosis probably do not take any active part in the formation of the toxic neutrophils (stab forms) seen in the peripheral blood in this disease.



Fig. 4.—Bone marrow (concentration technique) from a case of infectious mononucleosis showing neutrophilic myelocytes and neutrophilic metamyelocytes; the latter reveal normal development with an absence of toxic granules in the cytoplasm. Note the absence of lymphocytes in the bone marrow. Heterophile antibody titer was 1:1792.

Except for an increased number of eosinophilic myelocytes and metamyelocytes in the bone marrow in one case which was reflected by an eosinophilia (mature cells) in the peripheral blood of 16 per cent, all the other marrows showed a normal number of eosinophiles. The basophiles were reduced in number. The mature neutrophils averaged 7.9 per cent of the marrow granulocytes. This is approximately one-third the number seen in normal bone marrows and is due in a large part to the increase in the number of neutrophilic myelocytes.

The erythroid tissue was normal, except in the patient with the microcytic hypochromic anemia in whom the bone marrow revealed a normoblastic hyperplasia. In several of the marrows, groups or clusters of normoblastic elements were observed which is indicative of erythroid stimulation.

The megakaryocytes were increased in number in fifteen cases and normal in number in ten cases of infectious mononucleosis. The megakaryocytic increase was pronounced in those marrows with a myeloid hyperplasia. The maturation dispersion of the megakaryocytes was normal.

In a case of infectious mononucleosis in a girl 26 years of age with diabetes mellitus and not included in this group of twenty-five cases, the histiocytes or reticulum cells were increased in number and normoblastic clusters with frequent mitotic cells were seen scattered among the hyperplastic myeloid tissue. The reticulum cells appeared morphologically normal, and there were no indications that these elements were developing into specific hemopoietic elements such as myeloid, erythroid, megakaryocytes, plasma cells, and normal or abnormal lymphoid cells.

Plasma cells were only rarely seen in the marrow in our cases of infectious mononucleosis. In a 25-year-old woman with pulmonary tuberculosis who had immature lymphoid cells with basophilic cytoplasm in the peripheral blood, the bone marrow contained an increased number of normal and atypical types of plasmacytoid cells, some of which approached the Türk type with a leptochromatic structure of the nucleus. Cells of this type were also seen in the peripheral blood.

The number of lymphoid cells ranged between 4 to 24 per 100 bone marrow cells with an average of 10 per cent for the twenty-five cases. The greater number of lymphoid cells was seen in those marrows in which there was a lack of myeloid hyperplasia and in which admixture with peripheral blood was more pronounced than normal as indicated by the increased number of mature neutrophils. This latter finding with an increased number of lymphoid cells is always a definite indication of peripheral blood dilution and means that the bone marrow sample is only partially representative of the bone marrow pattern. The single or groups of lymphocytes seen in the bone marrow were similar to those seen in the peripheral blood. There were no morphologic indications from either the nucleus or cytoplasm that any of the marrow elements including those of the histiocytic system (reticulum cells) were giving rise to the normal or atypical lymphoid elements. In the marrows with a marked myeloid hyperplasia, the lymphocytes were decreased in number when compared with normal marrows in which the average number of lymphocytes is 17.8 per 100 bone marrow cells with a range of 12 to 26 per cent.

The myeloid-erythroid ratio averaged 3.0:1.0 with a reversal of the ratio in the marrow with the normoblastic hyperplasia (1.1:1.0) and a microcytic and hypochromic anemia in the peripheral blood; there was an increased myeloid ratio (6.69:1) in those marrows with a granulopoietic hyperplasia. The normal myeloid-erythroid ratio in our control cases was 2.75:1.0.

TABLE I. FINDINGS IN BLOOD IN INFECTIOUS MONONUCLEOSIS, NORMALS, CHRONIC LYMPHOCYTOSIS, WHOOPING COUGH, AND MEASLES

	HEMOGLOBIN (G./100 C.C. BLOOD)	ERYTHROCYTES (MIL- LONS)	LEUCOCYTES (THOU- SANDS)	PLATELETS (THOU- SANDS)	HEMATOCRIT (ERYTHROCYTES) (%)	HEMATOCRIT (LEUCOCYTES) (%)	SEDIMENTATION RATE (UNCORRECTED) (MM./ HOUR)	SEDIMENTATION RATE (CORRECTED) (MM./ HOUR)	MEAN CORPUSCULAR VOLUME (C.C.)	MEAN CORPUSCULAR HEMOGLOBIN (MICROGRAMS)	MEAN CORPUSCULAR HEMO- GLOBIN CONCENTRATION (%)	RETICULOCYTE COUNT	IGTERRUS INDEX	NEUTROPHILIC METAMYELOCYTES (%)	STAB FORMS (NEUTROPHILS) (%)	POLYMOIPHONUCLEAR NEUTROPHILS (%)	LYMPHOCYTES (%)	MONOCYTES (%)	EOSINOPHILS (%)	BASOPHILS (%)
Infectious mono- nucleosis (aver- age, 25 cases) Range	13.54 8.5 16.5	4.66 3.10 6.56	10.9 4.5 22.2	Normal Normal to slight in- crease	41.3 32.0 49.5	0.75 0.5 1.5	26.1 4.0 64.0	20.4 2.0 42.0	86.8 49.0 98.0	29.5 13.0 36.0	33.2 26.0 37.0	0.7 0.3 1.2	6.8 3.0 15.0	0.12 0.0 2.0	8.52 0.0 38.0	19.3 3.0 50.0	64.0 37.0 82.0	5.86 0.0 12.0	1.48 0.0 16.0	0.72 0.0 4.0
Normals (10*)	14.9	5.0	7.45	Normal	44.6	0.5	10.5	7.3	88.5	29.5	33.5	0.3	6.5	0.0	5.1	51.5	29.5	8.9	1.4	0.6
Chronic lympho- cytosis (2)	12.45	3.98	19.22	Normal	36.7	1.0	38.5	19.5	93.0	31.0	33.5	2.5	6.2	0.0	38.5	58.0	0.5	2.0	1.0	0.0
Whooping cough (1)	10.5	4.37	17.85	Normal	32.0	1.0	28.0	5.0	73.0	24.0	32.0	0.3	5.0	0.0	1.0	5.0	86.0	6.0	1.0	1.0
Measles (1)	16.5	5.56	7.1	Normal	52.0	0.5	13.0	12.0	93.0	29.0	31.0	1.0	5.0	0.0	35.0	26.0	24.0	11.0	2.0	2.0

*Number of cases studied.

TABLE II. BONE MARROW FINDINGS IN INFECTIOUS MONONUCLEOSIS, NORMALS, CHRONIC LYMPHOCTOSIS, WHOOPING COUGH, AND MEASLES

	MYELOID-ERYTHROID VOLUME	FAT VOLUME	MEGAKARYOCYTES	NUCLEATED MARROW CELLS	MYELOBLASTS	PROMYELOCYTES*	NEUTROPHILIC MYELOCYTES	EOSINOPHILIC MYELOCYTES	NEUTROPHILIC METAMYELOCYTES	EOSINOPHILIC METAMYELOCYTES	POLYMORPHONUCLEAR NEUTROPHILES	EOSINOPHILES	BASEOPHILES	PRONORMOBLASTS	BASEOPHILIC NORMOBLASTS	POLYCHROMATOPHILIC NORMOBLASTS	ORTHOCHEMATIC (ACIDOPHILIC) NORMOBLASTS	MYELOID-ERYTHROID RATIO	LYMPHOID CELLS IN MARROW (PER 100 CELLS)
	(%)	(%)	(PER 18 MM. SQUARE)	(THOUSANDS PER 0.1 MM. CUBE)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%) AND RATIO	(PER 100 CELLS)
Infectious mono- nucleosis (aver- age 25 cases)	11.1	1.0	Normal	4.30	0.44	1.10	24.8	1.96	61.34	1.81	7.4	1.01	.08	2.1	8.32	87.58	2.0	75:25	10.0
Range	5.0	0.0	Normal	210.0	0.0	0.0	0.0	0.0	32.0	0.0	2.0	0.0	0.0	1.0	2.0	76.0	0.0	56:44 (1.3:1)	4.0
	35.0	5.0	Increase	670.0	3.0	4.0	53.0	10.0	81.0	7.0	26.0	3.0	2.0	10.0	17.0	96.0	7.0	87:13 (6.69:1)	21.0
				(18 cases)															
Normals (101)	6.8	3.2	52.2	300.0	0.1	0.9	10.8	2.0	57.8	3.0	23.1	1.5	0.5	2.6	13.2	78.3	5.9	73:25: 26:65 (2.75:1)	17.8 (12 to 26)
Chronic lympho- cytosis (2)	5.5	3.5	Normal	196,000	0.5	1.0	19.5	5.0	59.5	3.0	9.0	2.0	0.5	5.0		79.5	2.0	62.5:37.5 8:1	22.0
Whooping cough (1)	8.0	0.0	Normal	110.0	1.0	1.0	35.0	1.0	51.0	5.0	5.0	1.0	0.0	1.0	1.0	94.0	1.0	85:15 5.6:1	20.0
Measles (1)	5.0	2.0	Increase	230.0	0.0	1.0	25.0	3.0	63.0	1.0	4.0	2.0	0.0	1.0	14.0	85.0	0.0	65:35 (1.85:1)	5.0

*Includes leucoblasts.

†Number of cases studied.

TABLE III. SUMMARY OF CONDITIONS STUDIED

	PERIPHERAL BLOOD		BONE MARROW STUDIES				
	LEUCOCYTES (THOUSANDS)	LYMPHOID AND "BLAST" CELLS (%)	MYELOID-ERYTHROID VOLUME (%)	CELL COUNT (THOUSANDS)	MYELOCYTES (%)	NEUTROPHILES (%)	LYMPHOID AND "BLAST" CELLS (%)
Normal controls (10*)	7.45	29.5	6.8	300.0	10.8	23.1	17.8
Infectious mononucleosis (25)	10.9	64.0	14.4	430.0	24.8	7.9	10.0
Whooping cough (1)	17.85	86.0	12.0	410.0	35.0	5.0	20.0
German measles (Rubella) (1)	7.1	24.0	5.0	230.0	8.0	4.0	5.0
Chronic lymphocytosis (2)	28.5	54.5	5.5		19.5	11.0	22.0
Chronic lymphatic leucemia (14)	14.2	77.0	Hyperplastic				50 and higher
Acute and subacute lymphatic leucemia (24)	11.67	39.0	Hyperplastic				Marked (over 100)
Acute leucemia (atypical types) (8)	10.4	50.0	Hyperplastic				Marked (over 100)

*Number of cases studied.

Besides the "normal" controls, the blood and bone marrow was studied in cases of whooping cough, German measles, chronic nonleukemic lymphocytosis, chronic lymphatic leukemia, acute and subacute lymphatic leukemia, and also "atypical" types of leukemia. These findings are recorded in Tables I to III.

CHRONIC NONINFECTIOUS LYMPHOCYTOSIS

Three cases of chronic lymphocytosis were studied in two men, one 46 and the other 48 years of age, and a woman 42 years of age. The average blood and bone marrow studies in the men are recorded in the tables. The woman presented similar clinical and hematologic findings. In these cases it was not during a routine blood examination that a lymphocytosis consisting of normal small lymphocytes comprised a large part of the differential count. There was no lymphadenopathy nor splenomegaly. The bone marrow of the woman patient studied showed a slight hypoplasia, quantitatively, with a myeloid-erythroid volume of 3 per cent. The myeloid, erythroid, and megakaryocytic elements showed all the phases of normal development, and 50 lymphocytes were counted among 100 nucleated bone marrow cells. The two cases recorded in the table have been observed for over seven years and have not showed any clinical hematologic changes. It is difficult to classify these cases. They may represent early cases of chronic lymphatic leukopenic leukemia or cases of chronic benign lymphocytosis. Only continued clinical and hematologic observations will finally reveal the diagnosis.

WHOOPIING COUGH

A case of whooping cough in a boy 6 years of age was studied hematologically. The findings in the blood and bone marrow are recorded in the table and it will be noted the anemia is of a simple microcytic type. In spite of marked lymphocytosis in the peripheral blood, the bone marrow reveals approximately the same number of lymphocytes found in the normal bone marrow.

The erythroid and megakaryocytic tissues were normal and myelopoiesis was of a toxic type.

GERMAN MEASLES (RUBELLA)

In a case of German measles in a medical student 26 years of age, the peripheral blood studies with the exception of the blood film were normal. The marked toxic shift to the left in the Schilling index with the presence of 6 plasma cells among 100 leucocytes was the most significant abnormality in the peripheral blood. With the exception of a slight increase in the number of megakaryocytes and a moderate toxic type of granulopoiesis, the bone marrow was normal. The number of lymphocytes in the bone marrow was decreased. No plasma cells were observed in the marrow in spite of these frequent findings in the peripheral blood. The significance of plasma cells in various infective conditions has been previously noted.³⁵

LEUCEMIA

Forty-six cases of leucemia were studied as controls (see Table III). In chronic lymphatic leucemia, irrespective of the quantitative or qualitative lymphocyte count in the peripheral blood, the bone marrow revealed a moderate to marked lymphocytic replacement of the bone marrow, depending upon the stage and duration of the disease. In the subacute and acute cases of lymphatic leucemia as well as stem cell, myeloid, monocytic, and atypical types, the bone marrow was always filled with proliferative types of blast cells which replaced the normal bone marrow elements (Fig. 5). This resulted in the neutropenia, anemia, and thrombopenia in the peripheral blood. The findings in the marrow were observed whether the leucocyte count in the peripheral blood was normal, subleukemic (leucopenic), or increased.

DISCUSSION

The diagnosis of infectious mononucleosis is based on three essential factors: (1) the clinical picture, (2) the serologic reaction, and (3) the hematologic studies, especially cytologic study of the blood. The presence of a relative large number of atypical "leucocytoid" lymphocytes is the most constant and, therefore, the most useful diagnostic sign of the disease.

Anemia does not occur in uncomplicated cases. The finding of a normocytic anemia in several of our cases and the presence of an iron deficiency anemia in one of our cases in all probability existed prior to the infectious mononucleosis. The toxic process associated with the disease is of too short duration to produce a toxic anemia. The study of the erythroid elements in the marrow of our cases with anemia was that seen in long-standing anemia states. In some of the cases without an anemia in the peripheral blood, a few clusters of normoblastic tissue were seen which was indicative of an erythropoietic response to an acute toxic condition. These normoblastic clusters in the bone marrow were of infrequent occurrence, in contrast to the frequent and widespread areas of erythropoietic activity seen in chronic anemia. The platelet count was normal

in most of the cases and moderately increased in several of the other cases of infectious mononucleosis. A low platelet count was not seen in our cases, and the bone marrow megakaryocytes were normal or increased in number with normal morphologic characteristics.

The abnormal cells which characterize the disease have been previously described. It is generally agreed that the cell is a benign type of lymphocyte which has its origin from the reticulum of the lymphatic tissue anywhere in the body. Apparently, the reticulum of the bone marrow does not take any active part in the formation of the atypical lymphocytes seen in this disease. It may be

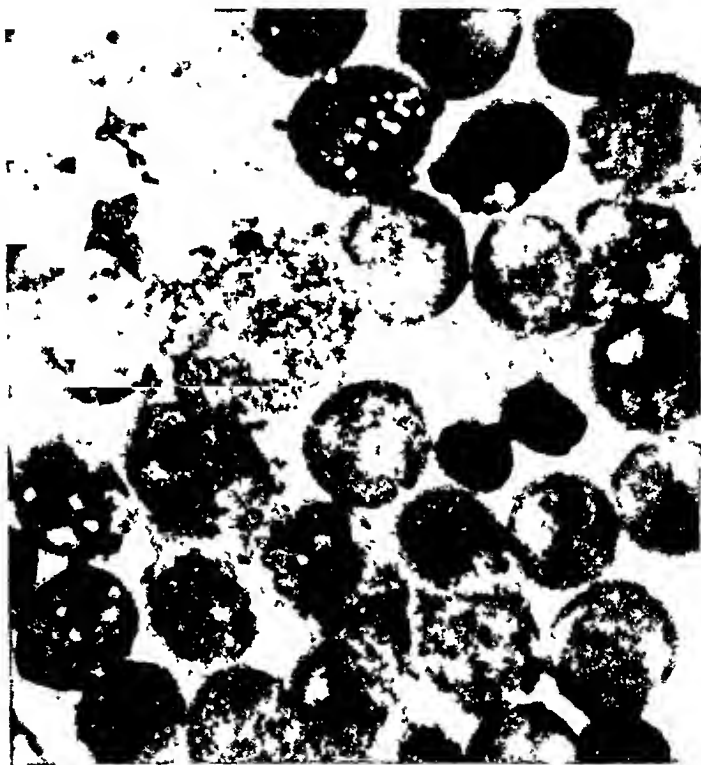


Fig. 5—Bone marrow (concentration technique) from a case of acute leucopenic myelosis showing the marrow filled with myeloblasts which have replaced the granulopoietic, erythropoietic, and megakaryocytic tissue.

that the products of the proliferative granulocytic cells in the bone marrow act as a protective mechanism during the toxic and neutropenic phase of the disease. Although little is known concerning the function and the potentialities of abnormal lymphocytes seen in the blood in cases of glandular fever, they lack the metastatic or replacement characteristics of the pathologic lymphocytes seen in the malignant lymphomas and leucemia. In infectious mononucleosis which is associated with a large number of atypical lymphocytes in the peripheral blood, the bone marrow shows a lack of lymphocytic infiltration and replacement of the marrow by lymphocytes seen in cases of lymphatic leucemia.

A number of cases of infectious lymphocytosis³⁶ have been reported in which there was a marked total leucocytosis with a high relative lymphocytosis and without clinical signs or symptoms. Leucocyte counts as high as 120,000 and differential counts with 97 per cent lymphocytes occurring in children have been reported. In several cases the bone marrow revealed many lymphocytes. There is no assurance that the presence of the lymphocytes in the bone marrow was not due to admixture with peripheral blood. The peripheral blood in these cases may resemble that seen in chronic lymphatic leucemia, but the bone marrow lacks the replacement or infiltration characteristic of lymphatic leucemia.

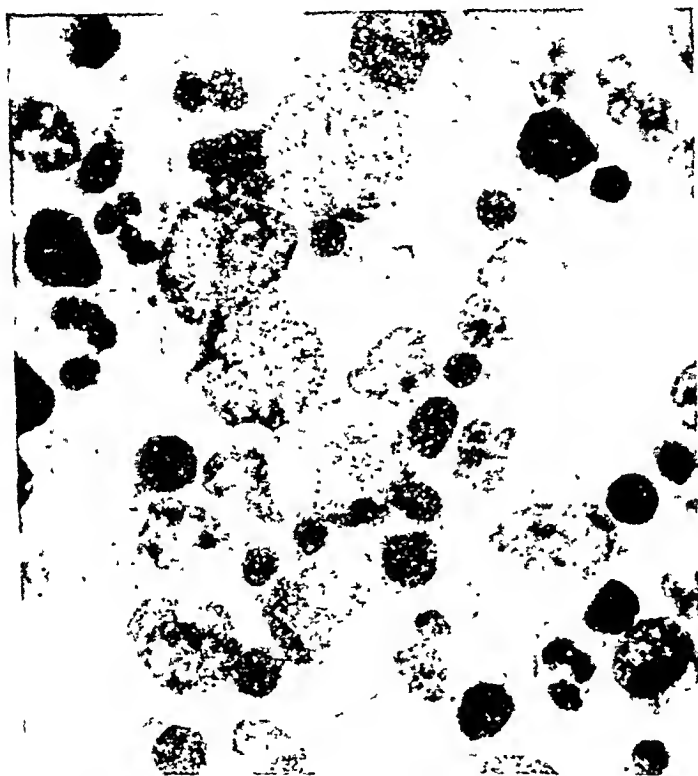


Fig. 6.—Bone marrow (concentration technique) from a case of acute infectious lymphocytosis showing moderate myeloid immaturity, normoblastic activity, and a few mature lymphocytes. The leucocyte count in the peripheral blood was 60,000 with a differential count consisting of 80 per cent small normal lymphocytes. Heterophile antibody reaction was negative.

In several of these cases studied by Limarzi and Poncher,³⁷ the bone marrow was hyperplastic and resembled the marrow of infectious mononucleosis (Fig. 6). Thus, bone marrow studies will immediately separate a malignant lymphocytic condition from a benign or nonleukemic reaction. It is of interest that the lymphocytes observed in the acute and chronic benign lymphocytosis do not possess the potentialities of transforming into macrophages as observed by Kolouch³⁸ in his experimental work on the lymphocytes in acute inflammation. Apparently, normal lymphocytes and atypical lymphocytes seen in lymphocytic reactions possess none of the malignant or transformation potentialities of

either leucemic cells or lymphocytes seen in acute inflammation; yet, leucemic and nonleucemic lymphocytes may appear morphologically similar. The pathologic, physiologic, and potential differences between benign and malignant types of lymphocytic elements are of interest in view of the recent work of Stedman²⁹ on the chemistry of the cell nuclei and the studies of Ehrlich and Harris⁴⁰ on the role of the lymphocytes in relationship to antibody formation. Malignancy or malignant cells produced by very diverse causes differ from the normal chiefly, if not entirely, by the acquisition of the capacity for uncontrolled growth. According to Stedman any agent which interferes with the chemical nature of the nucleus, that is, which causes the histone content of the nucleus of a cell to fall below a certain unknown level, will render that cell malignant unless some mechanism is present to restore histone to the nucleus. Whether this line of investigation can be carried to explain the pathologic physiologic differences between normal lymphocytes, the atypical lymphocytes of infectious mononucleosis, and the malignant lymphocytes of leukemia is an interesting speculation. It has been suggested by the work of Ehrlich and Harris⁴⁰ that the macrophages, the granular leucocytes, and the normal lymphocytes should be regarded as a team of substantially equal importance whose coordinate activities are essential for effective defense against infection. The granulopenia and the presence of atypical lymphocytes which may lack the power of formation of antibodies which are present in normal lymphocytes⁴¹ and protect the body from infection may be the cause of the toxic reaction seen in infectious mononucleosis. The toxic agent would then act on the bone marrow to produce a so-called maturation arrest and resultant myeloid hyperplasia; or the suppression of their delivery (release mechanism) to the peripheral blood results in a myeloid hyperplasia.

CONCLUSIONS

1. In spite of the large number of atypical lymphocytes in the peripheral blood in infectious mononucleosis the bone marrow is not involved.

2. The bone marrow in infectious mononucleosis reveals a myeloid hyperplasia and immaturity.

3. Either the "toxic agent" acts on the bone marrow to produce a myeloid hyperplasia and suppresses their delivery to the peripheral blood or the suppression of the myeloid tissue results in a myeloid hyperplasia.

4. The atypical lymphocytes in infectious mononucleosis show none of the metastatic or replacement characteristics of leukemia cells. In contrast, cases of leukemia with many abnormal cells in the peripheral blood, the bone marrow reveals varying degrees of replacement of the normal marrow by leucemic cells. The replacement characteristic of leucemic cells in lymphatic leukemia is seen in the bone marrow when the blood count is leucopenic, normal, or increased in type.

5. Sternal puncture is of diagnostic aid in differentiating benign and malignant types of lymphocytosis.

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PRESERVATIVES FOR SYPHILITIC SERUM WITH SPECIAL REFERENCE TO THE USE OF MERTHIOLATE

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MANY serologists prefer to receive whole blood specimens for the performance of serodiagnostic tests for syphilis. Others request serum, when specimens must be shipped from a distant point, in order to eliminate hemolysis and decomposition of the blood, especially during summer temperatures. Contamination of such separated serum frequently occurs, as it is not always practicable to separate serum under sterile conditions, and unsatisfactory, anticomplementary, or less accurate serologic tests often result. Preservative substances may be added to the sera to inhibit bacterial growth, but these must not alter syphilitic reagin nor affect specificity. Preservatives have been employed in serum samples sent out for special serologic surveys¹ or for serodiagnostic evaluation studies, without general knowledge of specific data concerning the effects of preservation and of the aging of sera.

Merthiolate (sodium ethyl mercuri thiosalicylate)[†] was recommended² as a bacteriostatic agent in spinal fluid specimens in 1:1,000 to 1:10,000 concentrations, and it has been used by the authors for more than two years with notable success in spinal fluids sent by mail from a seven-state area. Crawford and Hertert³ preserved sera for Wassermann tests by adding from 1.3 to 4.0 mg. per cubic centimeter of sulfanilamide, with no change in test reaction in eighteen days at room temperature and in observation of over 3,000 sera in duplicate. Perstein and Fishback⁴ preserved Wassermann positive sera with 50 per cent glycerine, and when thus treated the reagin was found to survive prolonged heating at 56° centigrade. Rouslaeroix and associates^{5, 6} added 8-hydroxyquinoline sulfate (sunoxol) in 1:5,000 concentration to preserve whole blood for the Wassermann test.

A number of substances have been used as preservatives for immune sera and for biologic products. Marshall⁷ reviewed and compared, from the standpoint of bacterial growth inhibition, some of the previously used serum preservatives. The following concentrations he found most favorable for bacteriostasis: trieresol, 0.2 per cent; 8-hydroxyquinoline sulfate (chinosol), 1:5,000; merthiolate, 1:5,000; glycerine, 1 per cent; and formalin, 0.2 per cent. Formalin had a destructive effect on serum antibodies, and the chinosol was not a good preservative. Boyd⁸ found that toluene was not as satisfactory as the aeriflavine or brilliant green dyes for preserving blood grouping sera. Jamieson and Powell⁹ used merthiolate in vaccines, toxins, toxoids, bacteriophages, and sera and found minimum injury to labile antigen and antibody fractions. Its high

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[†]Throughout this article the term Merthiolate is used repeatedly and is so written, referring in each instance to this compound manufactured by Eli Lilly and Company, Indianapolis, Ind.

degree of solubility, freedom from precipitation and coagulation characteristics, as well as its germicidal effectiveness in protein solutions, allowed it to be particularly useful in materials of a labile nature affected by the usual preservatives. Morgan and co-workers¹⁰ and Povitsky and Eisner¹¹ found merthiolate superior to phenol in the maintenance of the immunization properties of diphtheria toxoid. Waller¹² noted that merthiolate destroyed anti-Rh factors but not normal isoagglutinins in anti-Rh serum. Pressman and Grossberg¹³ showed that it interfered with the specific precipitation of antisera against beef or sheep serum coupled with diazotized p-amino benzoic acid. Simmons and Gentzkow¹⁴ noted that sodium fluoride may be used to preserve whole blood specimens sent by mail for chemical tests, the preservative effect being due to the inhibition of enzyme action.

The present study was undertaken to compare the preservative effect of those substances mentioned in the literature, with special reference to any interfering action on syphilitic reagin, and to include others which seemed worthy of trial. It was also planned to check more carefully the efficiency of the substances which gave the best preliminary results and to determine their desirability for routine use in sera mailed to a central laboratory for serologic testing.

EXPERIMENTAL

Comparison of Preservatives.—In addition to the preservative substances cited in the references, the following were used: sulfo-merthiolate (sodium p-ethyl mercuri thiosulfonate),* phenylmercuric borate, phenylmercuric nitrate, rivanol (ethoxydiamino-acridine),† zephiran chloride,‡ compound one (carboxymethoxylamine hemihydrochloride),‡¹⁵ penicillin, chloroform, and thymol.

The preliminary tests were performed by adding nineteen different preservatives, in a presumably optimum range of concentrations, to 5 c.e. samples of a Seitz filtered pool of positive reacting syphilitic sera. The 5 c.e. samples of sera were distributed in 9 c.e. nonsterile vials with vinylite lined screw caps, as bacterial contamination was desired to test the maximum effectiveness of the preservatives. Including the control serum not containing a preservative, thirty-eight trials were set up in duplicate. The two groups were read by two serologists, and the tabular results are the average of their observations. When possible the preservative substances, diluted with physiologically normal saline, were added to the vials in 0.1 c.e. amounts, the original concentration of each being at such a level that upon the addition of the 5 c.e. serum sample the final desired dilution was attained; that is, 0.1 c.e. of 5.1 per cent merthiolate in 0.85 per cent saline was added to the vial for the 1:1,000 concentration. It was necessary to weigh out carefully into the test vials the phenylmercuric salts, rivanol, thymol, sodium fluoride, and sulfanilamide, because of their insolubility; and in these instances 0.1 c.e. of saline was added to the vials and to the control serum specimen for comparable effect. Phenol was dissolved in 95 per cent ethanol. Glycerine in 50 per cent concentration diluted the serum, so that quantitative Kahn test titers were multiplied by two to give comparable figures.

*Furnished by courtesy of Eli Lilly and Company, Indianapolis, Ind.

†Furnished by courtesy of Winthrop Chemical Company, Inc., New York, N. Y.

‡Furnished by courtesy of Dr. C. B. Favour, Peter Bent Brigham Hospital, Boston, Mass.

Standard and quantitative Kahn tests were performed on the serum pool at the beginning of the trials and on a 1 c.c. portion withdrawn from the prepared samples at intervals of 4, 7, 10, and 17 days. The preserved samples were examined macroscopically for contamination at each test interval, and stained smears of each serum were examined at the end of the seventeen days. An average of the duplicate test quantitative Kahn reactions, and of the relative degrees of contamination of the sera, is shown in Table I, together with concentra-

TABLE I. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 80 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION				
		4 DAYS	7 DAYS	10 DAYS	17 DAYS	MACROSCOPIC				MICRO- SCOPIC 17 DAYS
						4 DAYS	7 DAYS	10 DAYS	17 DAYS	
Control serum		80	40	40	20	2+	2+	2+	4+	4+
Merthiolate	1:1,000	80	80	80	40	±	±	±	±	-
Merthiolate	1:2,500	80	80	80	40	±	±	±	±	-
Merthiolate	1:5,000	80	80	80	40	±	1+	2+	1+	2+
Sulfo-merthiolate	1:1,000	80	40	40	40	±	±	±	±	±
Sulfo-merthiolate	1:2,500	80	80	80	40	±	±	±	±	-
Sulfo-merthiolate	1:5,000	80	80	40	40	1+	1+	2+	2+	3+
8-Hydroxyquinoline sulfate	1:1,500	80	80	80	40	1+	1+	1+	1+	±
8-Hydroxyquinoline sulfate	1:5,000	80	80	80	40	2+	2+	2+	2+	3+
8-Hydroxyquinoline sulfate	1:10,000	80	80	40	40	2+	2+	2+	2+	3+
Phenylmercuric borate	1:1,250	80	80	40	40	±	±	±	±	±
Phenylmercuric borate	1:5,000	80	80	80	80	±	±	±	±	±
Phenylmercuric borate	1:62,500	80	80	80	80	2+	2+	3+	4+	4+
Rivanol	1:10,000	80	80	120	40	2+	1+	2+	2+	4+
Rivanol	1:20,000	80	80	80	40	2+	2+	2+	2+	4+
Zephiran	1:25,000	80	80	40	40	2+	2+	2+	4+	3+
Zephiran	1:50,000	80	120	40	40	2+	1+	2+	4+	3+
Compound one	.25 mg./ c.c.	80	80	80	80	2+	1+	2+	2+	4+
Penicillin	1 unit/c.c.	80	80	40	20	2+	1+	3+	4+	4+
Penicillin	2 units/c.c.	80	80	80	40	2+	1+	2+	4+	4+
Chloroform	.06 c.c.*/ 5 c.c.	40	40	40	40	±	±	±	±	-
Chloroform	.12 c.c./ 5 c.c.	4	4	20	40	2-	1+	1+	±	±
Thymol	10 mg./c.c.	0	0	0	0	Digested protein				-
Sodium fluoride	2 mg./c.c.	80	80	40	40	2+	2+	2+	2+	2+
Sodium fluoride	10 mg./c.c.	40	40	40	80	1+	1+	1+	±	-
Sulfanilamide	1.5 mg./c.c.	80	80	80	80	1+	1+	1+	1+	2+
Sulfanilamide	4 mg./c.c.	80	80	80	40	1+	±	±	±	±
Phenol	0.3%	40	40	40	40	±	1-	1+	2+	±
Phenol	0.5%	40	20	20	20	±	±	±	±	-
Formalin	0.2%	0	0	0	0	Insoluble precipitate				-
Tricresol	0.2%	40	40	40	20	±	±	±	±	-
Glycerine	1%	80	80	80	80	2-	3-	3+	3-	-
Glycerine	50%	80	80	80	80	-	-	-	4+	3+
Toluol	.06 c.c./ 5 c.c.	80	40	80	80	±	1-	1+	±	-
Acridlavine	1:100,000	80	80	40	40	2-	1-	2+	3-	4+
Acridlavine	1:200,000	80	40	40	40	2-	2+	2+	3-	4+
Brilliant green	1:3,000	80	80	40	40	1+	2+	1+	1+	±
Brilliant green	1:30,000	80	80	80	40	2-	2-	2+	2+	2+

*Note: 1 drop considered equal to .06 cubic centimeter.

tions of preservatives used. Because of marked drop in quantitative Kahn reactions, the following preservatives were eliminated from further consideration: chloroform, thymol, phenol, formalin, and trieresol. The following were eliminated because of failure to suppress bacterial contaminants in the concentrations employed: zephiran, penicillin, and acriflavine. Brilliant green imparted what we considered an undesirable color to the serum, and glycerine was deemed impractical for routine use because of its diluting effect, although it gave excellent preservation in 50 per cent concentration; therefore, these two substances were also eliminated.

TABLE II. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 160 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
						MACROSCOPIC				CULTURAL			
		7 DAYS	10 DAYS	14 DAYS	21 DAYS	7 DAYS	10 DAYS	14 DAYS	21 DAYS	7 DAYS	10 DAYS	14 DAYS	21 DAYS
Control		120	80	80	20	2+	3+	4+	4+	4+	4+	4+	4+
Merthiolate	1:2,500	120	120	120	80	+	1+	2+	2+	2+	2+	2+	2+
Merthiolate	1:5,000	120	120	120	80	+	1+	2+	2+	2+	2+	2+	2+
Sulfo-merthiolate	1:2,500	120	80	80	80	+	1+	2+	2+	2+	2+	2+	2+
Sulfo-merthiolate	1:5,000	120	120	120	120	+	+	1+	2+	1+	2+	2+	2+
8-Hydroxyquinoline sulfate	1:1,000	120	120	120	80	+	+	+	1+	-	-	+	+
8-Hydroxyquinoline sulfate	1:2,000	160	120	160	80	-	-	+	1+	-	-	1+	+
Phenylmercuric borate	1:5,000	120	120	120	80	+	+	1+	1+	-	+	1+	+
Phenylmercuric nitrate	1:5,000	120	120	120	80	+	1+	1+	3+	-	1+	2+	+
Phenylmercuric nitrate	1:50,000	160	120	120	80	1+	1+	2+	2+	1+	2+	2+	3+
Rivanol	1:5,000	120	80	40	40	2+	3+	3+	2+	2+	2+	3+	3+
Compound one	2 mg./c.c.	160	80	120	120	2+	3+	1+	2+	2+	2+	2+	2+
Compound one	1 mg./c.c.	160	80	80	80	2+	1+	1+	2+	2+	2+	2+	2+
Sodium fluoride	5 mg./c.c.	160	160	120	120	+	+	1+	1+	-	+	1+	1+
Sodium fluoride	7.5 mg./c.c.	160	160	160	120	+	-	+	1+	-	-	+	+
Sodium fluoride	10 mg./c.c.	160	160	160	160	+	-	+	+	-	-	+	+
Sulfanilamide	4 mg./c.c.	160	160	120	80	2+	1+	2+	1+	2+	1+	1+	1+
Sulfanilamide	5 mg./c.c.	160	120	120	120	2+	1+	2+	2+	2+	1+	1+	1+
Toluol	.06 c.c./5 c.c.	160	120	80	120	+	+	-	+	-	-	-	+
Toluol	.12 c.c./5 c.c.	120	120	80	120	+	-	-	+	-	+	-	-

*Note: 1 drop considered equal to .06 cubic centimeter.

Ten preservatives were compared in positive serum for trial two, the range of dilutions making a total of twenty different tests in duplicate. Test intervals were at 7, 10, 14, and 21 days. In addition to observing macroscopic contamination, 0.1 c.c. of each serum was inoculated into 5 c.c. of nutrient broth at each test interval, and the relative degree of developing growth was read after forty-eight hours' incubation. Contamination and the quantitative Kahn reactions are shown in Table II. All substances showed some promise of a preservative effect, with the exception of rivanol, although certain dilutions of the substances were relatively ineffective, for example, phenylmercuric nitrate, 1:50,000.

The following six preservatives were compared with a control serum in trial three: merthiolate, 1:1,000 and 1:2,500; sulfo-merthiolate, 1:2,500; 8-hydroxyquinoline sulfate, 1:2,000; sodium fluoride, 10 mg. per cubic centimeter, 12.5 mg. per cubic centimeter, and 15 mg. per cubic centimeter; sulfanilamide, 5 mg. per cubic centimeter; and toluol, 1 drop (0.06 c.c. per 5 c.c.). Three series were set up, with positive reacting serum in sterile and nonsterile vials and with negative serum in nonsterile vials. Aseptic precautions were used with the "sterile" series. The results obtained are not tabulated, for all sera, including the controls, maintained 40 Kahn units for three weeks with the exception of the 1:1,000 merthiolate serum, which dropped to 20 units in two weeks for the nonsterile vials and to 20 units in three weeks for the sterile vials. The positive serum control showed 1 plus and 2 plus contamination in both series throughout. The 8-hydroxyquinoline sulfate serum showed plus-minus contamination in two weeks, and sulfanilamide serum plus-minus to 1 plus contamination in one to two weeks, and that with toluol allowed plus-minus to 1 plus contamination throughout. It was probable that the Seitz filtration did not completely sterilize the positive serum pool. In the negative serum trials, in nonsterile vials, the control serum and toluol-preserved serum showed 1 plus contamination. A nonspecific precipitate developed after two weeks in the 8-hydroxyquinoline sulfate serum.

At this point, sodium fluoride appeared advantageous. Vials were prepared by adding the required amount of 1:25 aqueous solution of the salt and drying in a warm temperature, so that concentrations of 5 mg. per cubic centimeter and 7.5 mg. per cubic centimeter in serum would be attained by adding 3, 4, or 5 c.c. of serum to the proper vials. Seventeen vials each of 5 mg. per cubic centimeter and 7.5 mg. per cubic centimeter concentration were tested with positive and negative sera. Sodium fluoride was shown to affect the specificity of the Kahn test in one week's time in 8 of 11 negative sera in 5 mg. per cubic centimeter vials and in all 14 of 7.5 mg. per cubic centimeter negative serum vials. Kahn reactions as high as 1 3 4 were obtained in the standard test.

In Table III is shown the effect of merthiolate in concentrations from 1:1,000 to 1:10,000, compound one at 1 mg. per cubic centimeter and sulfanilamide at 5

TABLE III. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 40 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
		1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	MACROSCOPIC				CULTURAL			
						1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS
Control		40	40	40	20	1-	1+	2-	4-	1-	4-	2+	2+
Merthiolate	1:1,000	10	10	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:2,500	40	40	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:5,000	40	40	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:7,500	40	40	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:10,000	80	10	40	40	-	-	-	-	-	-	-	-
Compound one	1 mg./c.c.	40	40	40	20	-	1-	2-	4-	1+	2-	2+	2+
Sulfanilamide	5 mg./c.c.	40	40	40	40	-	+	1-	2-	1+	1-	1-	1-

mg. per cubic centimeter on positive Kahn serum giving a titer of 40 Kahn units. Only the control serum and that containing compound one decreased to 20 Kahn units in four weeks' time. The control serum, that containing compound one, and that with sulfanilamide showed contamination.

Further comparisons were made of the effectiveness of merthiolate in 1:1,000, 1:2,500, and 1:5,000 concentrations, compound one at 2 mg. per cubic centimeter of serum, 8-hydroxyquinoline sulfate at 1:2,000, phenylmercuric nitrate and borate at 1:5,000, sulfanilamide at 5 mg. per cubic centimeter, and toluol at 1 drop per 5 cubic centimeter. Sulfo-merthiolate was not tested further, since it showed no advantages over merthiolate. A slightly different method of vial preparation was used for these comparisons, in that the preservatives were dissolved in water, except for the phenylmercuric salts which were made up 1:800 in 95 per cent ethanol (not completely soluble). The sulfanilamide was used in a 2 per cent solution in 95 per cent ethanol, and the toluol was added to the serum in liquid form. The sterile vials containing the preservatives, with the tops loosened, were placed in a desiccator until the diluent had evaporated.

TABLE IV. EFFECT OF TEST PRESERVATIVES IN VARYING CONCENTRATIONS ON POSITIVE SYPHILITIC SERUM, TWO RUNS AVERAGED (ORIGINAL KAHN REACTION, 40 KAHN UNITS)

SUBSTANCE	CONCENTRATION IN SERUM	QUANTITATIVE KAHN REACTIONS (KAHN UNITS)				CONTAMINATION							
		1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	MACROSCOPIC				CULTURAL			
						1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS
Control		40	20	4	4	4+	4+	4+	4+	4+	3+	3+	3+
Merthiolate	1:1,000	40	40	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:2,500	40	40	40	40	-	-	-	-	-	-	-	-
Merthiolate	1:5,000	40	40	40	40	-	-	-	-	-	-	-	-
Compound one	2 mg./c.c.	40	40	40	20	-	-	-	+	-	-	-	+
8-Hydroxyquinoline sulfate	1:2,000	40	40	40	40	±	Precipitate			-	-	-	-
Phenylmercuric borate	1:5,000	40	40	40	40	-	±	1+	2+	-	±	1+	1+
Phenylmercuric nitrate	1:5,000	40	40	40	40	1+	1+	1+	2+	±	±	±	1+
Sulfanilamide	5 mg./c.c.	80	40	40	40	-	±	±	±	-	1+	1+	1+
Toluol	.06 c.c./5 c.c.	80	40	40	20	2+	1+	2+	2+	1+	2+	2+	2+

Five cubic centimeters of the test sera were added to each vial and were mixed thoroughly. Two duplicate groups of positive sera (one of 40 and one of 20 units) were tested, as were three of negative sera. Tabulation of all results is not shown. In the positive serum of 20 units, all preservatives maintained the original 20 Kahn unit titer for four weeks, with only toluol allowing slight contamination. In the positive serum of 40 units, which was set up with a Seitz filtered serum that had previously shown gross contamination, phenylmercuric borate, nitrate, sulfanilamide, and toluol did not restrain bacterial growth; whereas, merthiolate completely inhibited growth (Table IV). Compound one and toluol allowed a quantitative Kahn decrease in titer from 40 units to 20 units in four weeks.

TABLE V. EFFECT OF MERTHIOLATE 1:2,500 ON KAHN REACTIONS OF SERA WHICH STOOD ONE, TWO, AND THREE WEEKS AT ROOM TEMPERATURE

ORIGINAL KAHN TEST REACTION GROUP*	CHANGE FROM ORIGINAL KAHN REACTIONS	ONE WEEK		TWO WEEKS		THREE WEEKS	
		NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT
Positive (4 Kahn units or above)	No change†	127	63.5	112	56.0	88	48.9
	Slight change‡	12	6.0	22	11.0	29(1)	16.1
	Significant change§	6	3.0	11(2)	5.5	10(2)	5.6
Positive (2 plus and 3 plus reactions)	No change†	28	14.0	29	14.5	26	14.4
	Slight change‡	7(5†)	3.5	5(3†)	2.5	7(2†)	3.9
	Significant change§	1	0.5	2(1)	1.0	3()	1.7
Doubtful (1 plus reaction)	No change†	14	7.0	10	5.0	8	4.4
	Slight change‡	5(4†)	2.5	9(7†)	4.5	9(8†)	5.0
Totals		200	100.0	200	100.0	180	100.0

Note: 23 negative sera were also included in this trial for the two weeks period and 19 for the three-week period. Four gave slight plus-minus or 1 plus reactions in single tubes on rare occasions but never sufficient to change the report to doubtful.

*Based on one reading, a 3-tube total of 5 to 12 plus is Positive; 2 to 4 plus, Doubtful; 0 to 1 plus, Negative.

†Reaction remained in same group, or quantitative test end point differed in not more than one dilution.

‡Positive to doubtful, doubtful to negative, or a quantitative end point difference of not more than three dilutions.

§Positive to negative or a quantitative end point difference of four dilutions, or more.

||Contaminated.

||No greater difference than 3 plus but sufficient to change the reported reaction.

In the three trials with negative sera, totaling sixty tests, the substances listed in Table IV did not affect the specificity of the Kahn test over a four-week period, although 8-hydroxyquinoline sulfate gave a confusing extraneous precipitate in two weeks in all instances. In two of these series, toluol allowed contamination to develop.

Practical Use of Merthiolate.—The preceding experiments demonstrated an apparent superiority of merthiolate; hence, it was chosen for further trial. Screw

TABLE VI. EFFECT OF MERTHIOLATE 1:1,000 ON KAHN REACTIONS OF SERA WHICH STOOD ONE, TWO, AND THREE WEEKS AT ROOM TEMPERATURE

ORIGINAL KAHN TEST REACTION GROUP*	CHANGE FROM ORIGINAL KAHN REACTIONS	ONE WEEK		TWO WEEKS		THREE WEEKS	
		NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT	NUMBER OF SERA	PER CENT
Positive (4 Kahn units or above)	No change†	39	48.1	33	40.7	29	36.7
	Slight change‡	19	23.5	19	23.5	22	27.9
	Significant change§	3	3.7	9	11.1	9()	11.4
Positive (2 plus and 3 plus reactions)	No change†	9	11.1	8	9.9	5()	6.3
	Slight change‡	4(†)	4.9	1†	1.2	1	1.3
	Significant change§	2	2.5	6	7.4	8	10.1
Doubtful (1 plus reaction)	No change†	2	2.5	2	2.5	2	2.5
	Slight change‡	3(2†)	3.7	3(2†)	3.7	3(2†)	3.8
Totals		81	100.0	81	100.0	79	100.0

Note: 12 negative sera were also included in this trial for the three-week period, one of which gave a false doubtful reaction in two weeks (±1) but a negative in three weeks (—1).

*Based on one reading, a 3-tube total of 5 to 12 plus is Positive; 2 to 4 plus, Doubtful; 0 to 1 plus, Negative.

†Reaction remained in same group, or quantitative test end point differed in not more than one dilution.

‡Positive to doubtful, doubtful to negative, or a quantitative end point difference of not more than three dilutions.

§Positive to negative, or a quantitative end point difference of four dilutions, or more.

||Contaminated.

||No greater difference than 3 plus, but sufficient to change the reported reaction.

cap vials of 9 c.c. capacity were autoclaved. Two series were so prepared that upon the addition of 3, 4, or 5 c.c. of serum the resultant concentrations of 1:2,500 and 1:1,000 were obtained. To accomplish this, six tests of these sterile vials were prepared containing, respectively, 0.1 c.c. of merthiolate prepared in 1:33.3, 1:25, 1:20, 1:83.3, 1:62.5, and 1:50 aqueous dilutions. Addition of 3, 4, or 5 c.c. of serum, respectively, to vials containing the first three concentrations

TABLE VII. SERA SHOWING SIGNIFICANT CHANGES IN KAHN TITER

SPECIMEN	ORIGINAL REACTION	ONE WEEK	TWO WEEKS	THREE WEEKS	CLINICAL NOTES
<i>1:2,500 Merthiolate</i>					
3	-23	--4	--2	---	Early latent syphilis; previous R
6	360 K.U.	200 K.U.	120 K.U.	120 K.U.	Neurosyphilis, asymptomatic; previous R
18	360 K.U.	360 K.U.	200 K.U.	200 K.U.	Gonorrhea; early latent syphilis; R at time of specimen
20	400 K.U.	240 K.U.	160 K.U.	160 K.U.	No record
26	344	---	---	-11	No record
55	±44	-22	--±	---	Gonorrhea; R at time of specimen; incomplete history
64	360 K.U.	200 K.U.*	200 K.U.*	200 K.U.*	Primary syphilis; R at time of specimen
67	160 K.U.	80 K.U.	---	---	Gonorrhea; primary syphilis; R at time of specimen
74	4 K.U.	244	-±*	---	Primary syphilis, untreated
81†	4 K.U.	---	---	---	False positive, incorrectly recorded as early latent; inductee, no R; titer fluctuated, 360 K.U. to negative within 20 days
92	-34	---	---	---	Latent syphilis, previous R; gonorrhea, R now
117	4 K.U.	4 K.U.	---	---	Early latent, completed second R 2 days before
148	20 K.U.	-44	-±1	-1	Early latent, no R; quantitative Kahn fluctuated, 4 to 40 K.U.
150†	4 K.U.	---	---	---	Same as Specimen 81
<i>1:1,000 Merthiolate</i>					
12	±24	-13	-12	-±	Late latent syphilis
26	344	---	---	---	No record
32	-44	112	---	-±	Late latent syphilis
55	±44	---	---	---	Same as Specimen 55 above
63†	4 K.U.	-±3	---	---	False positive due to smallpox vaccination month before
72†	4 K.U.	-11	---	---	Same as Specimen 63
81†	4 K.U.	---	---	---	Same as Specimen 81 above
83	200 K.U.	120 K.U.	20 K.U.	20 K.U.	Early latent syphilis, no R
92	-34	---	---	---	Same as Specimen 92 above
94	344	-1	-±	---	Questionable primary; observed one month, dismissed without R
132	4 K.U.	-23	---	---	No record
136	-44	-±	---	---	No record
142	-44	-23	-23	---	No record
148	200 K.U.	-44	-1	-1*	Same as Specimen 148 above
149	-44	-24	---	-1	Mucocutaneous relapse
150†	4 K.U.	---	---	---	Same as Specimen 81 above
152	-44	-±	---	---	Old syphilis, previous R; chaneroid or lymphogranuloma at time of specimen, no R

K.U., Kahn units.

R, Treatment.

*Contamination.

†Same patient.

‡Same patient.

gives a 1:1,000 concentration. Similarly, such quantities in the last three tubes gives a 1:2,500 concentration.

The blood samples were obtained in 30 c.e. vacuum venules from patients at the local venereal disease clinic. They were refrigerated until the sera were separated and placed in the vials containing merthiolate. In some instances, serum stood on the clot for as long as three days. The sera were then kept at room temperature in the dark, and 1 c.e. portions were removed immediately after adding the serum and at intervals of one, two, and three weeks, unless they were sooner exhausted. These portions were centrifuged, decanted, inactivated for thirty minutes at 56° C., and then tested by the Kahn standard and by the quantitative technique when indicated.

Results with the two series are shown in Tables V and VI. The original Kahn reactions were separated into a quantitative group showing 4 Kahn units or more, a positive group showing 2 or 3 plus reactions, a doubtful group, and a negative group. Changes at one, two, and three weeks were tabulated as showing "no change," "slight change," or "significant change" (see footnote criteria in Tables V and VI. The 1:1,000 merthiolate gave a greater percentage of significant changes than did the 1:2,500 concentration, and both series showed greater decreases in titer after two and three weeks than at one week. One negative serum (1:1,000 preservation) became doubtful (± 11) in two weeks but at the end of three weeks reverted to negative ($- - 1$). Otherwise there were no changes in specificity.

TABLE VIII. DIFFERENCES IN KAHN REACTIONS OVER TWO- OR THREE-WEEK PERIODS WITH SAME SERA AT TWO CONCENTRATIONS OF MERTHIOLATE (1:2,500 AND 1:1,000)

DIFFERENCE IN REACTIONS BETWEEN THE TWO	NUMBER OF SERA	NUMBER GIVING HIGHER TITER AT 1:2,500	NUMBER GIVING HIGHER TITER AT 1:1,000
Agreement	63	-	-
Slight disagreement	19	15	4
Significant disagreement	11	10	1
Totals	93	25	5

After three weeks, of the sera in the 1:1,000 series, 45.5 per cent showed "no change" in titer, 33 per cent showed a "slight change," and 21.5 per cent showed a "significant change." The sera in the 1:2,500 series gave the following at the end of three weeks: 67.7 per cent "no change"; 25 per cent "slight change"; 7.3 per cent "significant change." In Table VII are listed the cases with significant changes in the Kahn reaction, and notes as available are included.

It was possible to preserve ninety-three specimens in both concentrations. The results are summarized in Table VIII. The 1:2,500 dilution gave better results in a proportion of 5 to 1. In Table IX are listed the detailed results in those cases of significant disagreement.

Of 223 sera preserved by the 1:2,500 dilution, 14 showed contamination: 5 at one week, 7 at two weeks, and 2 at the end of the third week. Of 93 sera preserved by the 1:1,000 dilution only two specimens were contaminated.

TABLE IX. TABULATION OF DUPLICATE SERA SHOWING SIGNIFICANT DISAGREEMENT WHEN PRESERVED (1:2,500 AND 1:1,000 WITH MERTHIOLATE)

SPECIMEN	ORIGINAL REACTIONS		ONE WEEK		TWO WEEKS		THREE WEEKS	
	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000
32	-44	-44	134	112	123*	---	-34*	--±
39	20 K.U.	40 K.U.	40 K.U.	-24	40 K.U.	-12	40 K.U.	No test
63	4 K.U.	4 K.U.	-34	-23	-24	---	-2	---
72	4 K.U.	4 K.U.	-44*	-11	-23*	---	-13*	---
83	200 K.U.	200 K.U.	200 K.U.	120 K.U.	200 K.U.	20 K.U.	120 K.U.	20 K.U.
94	344	344	-44	-1	-44	--±	-34	---
123	---	---	---	-±	---	+11	---	-1
132	4 K.U.	4 K.U.	-44	-23	-24	---	-44	---
136	-44	-44	-24	-±	-23	---	-44	---
149	-44	-44	-24	-24	-44	---	-24	-1
152	-44	-44	-44	-±	-44	---	-44	---

K.U., Kahn units.

*Serum showed contamination.

DISCUSSION

Inaccurate or unsatisfactory Kahn reactions have undoubtedly resulted, in the past, from either distant shipment of plain sera or the use of various preservatives unknown to the serologist. It is possible that syphilitic serum loses reagin to some extent after it has been separated from the clot,¹⁶ and unless shipped as whole blood, or with a satisfactory preservative, undersensitive tests will be reported from the distant laboratory.

The present study indicates that none of the preservatives, in concentrations tested, shows a better preservative effect on syphilitic serum than merthiolate. Sulfo-merthiolate appeared to be almost as satisfactory. Compound one in a concentration of 2 mg. per cubic centimeter was useful in this application. Neither sulfanilamide, phenylmercuric borate or nitrate, nor toluol gave consistent bacterial inhibition in sera, although reagin content did not decrease appreciably in their presence. Crawford and Hertert's³ recommendation of sulfanilamide was not confirmed in our limited study. The phenylmercuric salts are impractical because of their low solubility. Glycerine, which in 50 per cent concentration preserved reagin, cannot be used routinely because of the high dilution required.

The use of sterile rather than nonsterile vials in the first part of the study did not prove advantageous, since bacterial contamination was desired to test the effectiveness of the preservatives. Either the vials or the serum furnished the contaminants in most instances, as would often occur in practical application of preservatives to serum.

Merthiolate was effective bacteriostatically in 1:1,000 to 1:10,000 concentrations. The 1:1,000 concentration gave a greater percentage of decreased Kahn titers and one nonspecific reaction; therefore, 1:2,500 is recommended for practical usage. Merthiolate 1:2,500 did not markedly interfere with reagin over a three-week period in 92.7 per cent of 180 positive sera. The drop in one week was less, 96.5 per cent of 200 sera remaining unchanged or dropping insignificantly. The comparative figure at one week's time for the 1:1,000 series was 93.8 per cent of 81 sera. Under present conditions of transport, sera should reach a laboratory within one week.

The seven cases (3.5 per cent of total) in the 1:2,500 series, which changed significantly in one week (see Table VII), would have given one false negative (Specimen 92), and it was contaminated. Three others (Specimens 26, 81, and 150) were from patients not known to have syphilis. The other three (Specimens 6, 20, and 64), though reduced in reagin, still gave strong positives. Of twenty-six cases showing significant reduction of reagin in Table VII, for which clinical data were available, nine were either receiving penicillin or had just completed such treatment at the time the blood was obtained. Undue significance must not be attributed to this, for many of the other cases in this study were likewise being treated or had just completed treatment, yet showed no decrease in reagin.

False positive sera may decrease in titer. Three specimens were obtained from a patient (Case 1) diagnosed as having false positive reactions due to recent immunizations and smallpox vaccination, also having a history of upper respiratory infection. Another patient (Case 2) had a false positive reaction due to a malaria attack two and one-half weeks previously. A third patient (Case 3), a 17-year-old inductee, incorrectly recorded as having early latent syphilis, denied exposure and had other quantitative fluctuations from 360 Kahn units to negative. These reactions are shown in Table X.

TABLE X. KAHN REACTIONS SHOWING DROPS IN TITER ASSOCIATED WITH FALSE POSITIVE REACTIONS FROM THREE PATIENTS

DATE	CASE	SPECI- MEN	ORIGINAL		ONE WEEK		TWO WEEKS		THREE WEEKS	
			1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000	1:2,500	1:1,000
1/31	1	58	20 K.U.	20 K.U.	444	244	-23	-14	-23	-23
2/5	1	63	4 K.U.	4 K.U.	-34	-13	-24	---	-2	---
2/7	1	72	4 K.U.	4 K.U.	-44	-11	-23	---	-13	---
12/21	2	16	±12	-12	-11	---	-11	---	---	---
2/12	3	81	4 K.U.	4 K.U.	---	---	---	---	---	---
2/27	3	150	4 K.U.	4 K.U.	---	---	---	---	---	---

K.U., Kahn units.

Five sera from both series showed very slight increases in titer after two weeks, one of which resulted in a nonspecific Kahn reaction (-11) in 1:1,000 merthiolate concentration, the only instance of the development of a false reaction with merthiolate.

Contamination either enhanced or decreased Kahn titers in sixteen instances. Given sufficient inoculum, 1:2,500 merthiolate will allow contaminants to increase, usually within two weeks. Tests on such sera should be carefully considered and rechecked with another specimen if possible.

The criteria for designating change in reaction as "no change," "slight change," or "significant change" were devised so that they would coincide with a physician's usual interpretation of such reports were he to receive them in following a case. A false drop in quantitative titer of three dilutions in a preserved sample might be misleading. Quantitative titers on preserved sera should be interpreted with care.

SUMMARY AND CONCLUSIONS

A study of the preservation of syphilitic reagin as demonstrated by the Kahn test is presented.

Twenty preservative substances were compared for their effect on positive syphilitic sera and on negative sera over periods of two and one-half to four weeks at room temperature. None gave greater preservation than merthiolate (sodium ethyl mercuri thiosalicylate), which was effective, depending on the amount of original contamination, in concentrations of 1:1,000 to 1:10,000. A comparison of two concentrations revealed that serum containing 1:2,500 merthiolate showed less drop in reagin content than that with the 1:1,000 amount. Test specificity was not affected when concentrations less than 1:1,000 were used.

A practical method of preserving sera with merthiolate 1:2,500 for shipment by mail is described. Less than 4 per cent of 200 sera so tested showed a significant loss in titer in one week's time, and only 7.3 per cent showed a significant decrease in three weeks. The use of such a method of preservation would result in more accurate serology in instances where whole blood cannot be sent to a laboratory without danger of gross deterioration. The method is not intended to supplant shipments of whole blood when such can be made satisfactorily.

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SECONDARY BACILLUS PYOCYANEUS INFECTION IN MENINGITIS FOLLOWING INTRATHECAL PENICILLIN THERAPY

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FROM time to time there have been reported in the literature cases of secondary meningeal infections caused by intrathecal punctures. In 1911, Schlagenhauser¹ reported five cases of *Bacillus pyocyaneus* meningitis resulting from contaminated saline used in mixing spinal anesthetics administered to these patients. A total of eleven cases of *B. pyocyaneus* meningitis after diagnostic lumbar punctures has been reported by Sonnenschein,² Levy and Cohen,³ Evans,⁴ and Wise and Musser.⁵ Kerman and associates,⁶ in 1943, reported a case of *B. pyocyaneus* meningitis following pneumoencephalogram. In this instance a wash bottle through which helium was passed was found to be the source of the contamination. In a review of the literature Kerman stated that among the fifty-two cases reported there was a mortality rate of 58 per cent, despite various types of therapy, including the sulfonamides.

During 1945, one of us (E. A.) observed four patients with pneumococcus meningitis who were treated with penicillin, intramuscularly and intrathecally, and subsequently developed a secondary *B. pyocyaneus* meningitis. Two of the patients were at the Willard Parker Hospital, where bacteriologic investigation established the source of the secondary infection.

CASE REPORTS

CASE 1.—Patient A. M., a 9-month-old Puerto Rican female, was admitted Dec. 16, 1944, with a diagnosis of pertussis because of a history of coughing and vomiting for six days. When first seen the patient had convulsions, the anterior fontanel was bulging, and dilated veins were prominent over the scalp. The temperature was 104° F.; pulse, 160; and respirations, 52. A spinal tap was done, and 10 c.c. of cloudy fluid were removed under increased pressure. On direct examination this fluid showed the presence of pneumococcus type XVIII.

Treatment with 10,000 units of penicillin intramuscularly every three hours and daily intrathecal injections of 10,000 units of penicillin in 10 c.c. of saline was started. Orally, 5 Gm. of sulfadiazine were given daily to maintain blood levels between 9 to 14 mg. per cent. After twenty-two days, because the spinal fluid cultures continued to show pneumococci, the dosage of the intrathecal penicillin was increased to 20,000 units in bi-daily injections and continued for twelve days.

There were many exacerbations in the course of this patient's illness, but as the temperature was normal from Jan. 10 to 19, 1945 (Fig. 1), intrathecal treatment was discontinued on the nineteenth. On January 22 the temperature rose, the patient again became irritable, and the anterior fontanel became tense. Pneumococcus type XVIII was again recovered from the spinal fluid, and the bi-daily intrathecal injections of penicillin were

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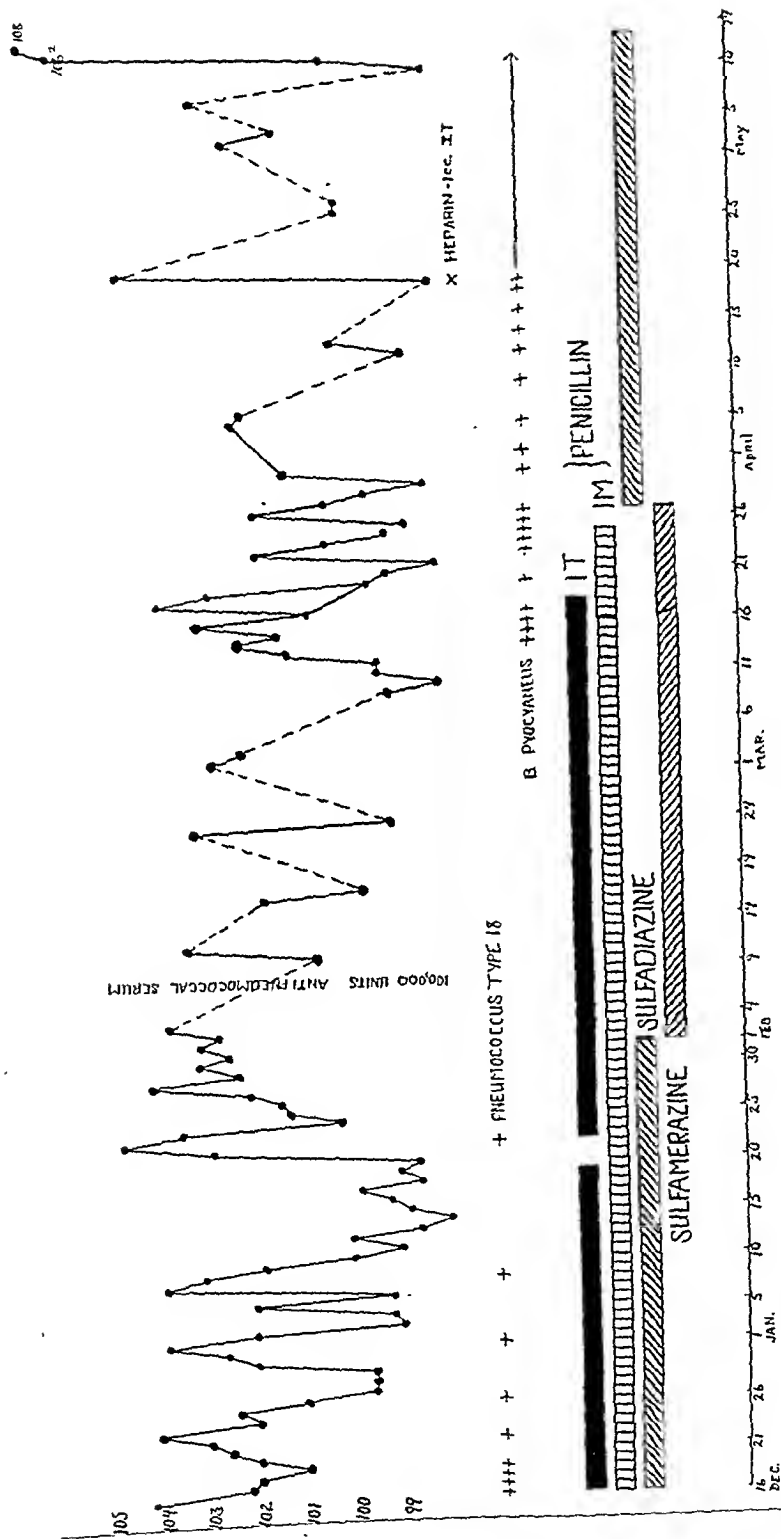


Fig. 1.—Case 1.

resumed. For the next three weeks the temperature ranged from 103 to 99° F., and signs of meningeal irritation persisted, even though cultures of the spinal fluid showed no growth after January 22. On February 8, 100,000 units of type XVIII antipneumococcus serum were given intravenously. The temperature elevation gradually decreased and was normal from March 7 to 13. During this period spinal fluid drainage was carried out, and intrathecal penicillin was administered every morning and in the evenings only when the fontanel was bulging.

On the evening of March 13, 1945, the patient vomited, and at 2 A.M. on March 14 the temperature rose to 102° Fahrenheit. The fontanel was bulging, eyes protruded, and the patient was irritable. Very thick purulent spinal fluid was obtained under increased pressure, and 10,000 units of penicillin were instilled after drainage. This treatment was continued until March 17 when a report was obtained that a gram-negative bacillus had been cultured from all specimens of spinal fluid from March 14. The bacillus was identified later as *B. pyocyaneus*. From March 18 to the day of death no intrathecal penicillin was administered, but bi-daily lumbar punctures were performed to relieve pressure and to drain off fluid. One cubic centimeter of heparin was given intrathecally to prevent adhesions. Intramuscular injections of penicillin were not given after March 25. A total of 1,580,000 units of penicillin intrathecally and 7,430,000 units intramuscularly were administered. No local abscesses were ever noted in the buttocks in the region of intramuscular injections.

The spinal fluid cultures were persistently positive for *B. pyocyaneus*, and the patient grew steadily worse and died on May 14, 1945.

CASE 2.—Patient, B. H., an 83-year-old white woman, was admitted Feb. 24, 1945, to another hospital, with the history of pain in the right ear for two days. A spinal tap was done and 20,000 units of penicillin intrathecally and 20,000 units intramuscularly were given. The patient was transferred to Willard Parker Hospital on the same day.

On admission she was unconscious and had a stiff neck, positive Kernig sign, and diminished deep reflexes. She was given an infusion of 5 per cent glucose in saline and sodium sulfadiazine intravenously. On February 27 it was reported that the spinal fluid and blood cultures showed pneumococcus type XIV, and treatment with 20,000 units of penicillin once daily intrathecally and 15,000 units intramuscularly every three hours was instituted. Sulfadiazine therapy was also continued.

The patient became rational on the third hospital day; the temperature gradually fell and reached normal on March 11, 1945. On March 14 at 2 A.M. the temperature again rose to 103.4° F. (Fig. 2), and the patient again became slightly irrational. There was marked stiffness of the neck and positive Kernig sign. At this time purulent spinal fluid was obtained under increased pressure.

On March 17 the laboratory reported that a gram-negative bacillus was cultured from all spinal fluid specimens since March 14 and that this microorganism was also a *B. pyocyaneus*. Drainage of the spinal fluid was done thereafter without injection of penicillin. The patient continued to receive sulfadiazine and intramuscular injections of penicillin. The temperature rose from 103 to 105° Fahrenheit. She became comatose, developed rales at the lung bases and scattered areas of bronchial breathing. She died on March 28, 1945. This patient received a total of 380,000 units of penicillin intrathecally and 2,680,000 units intramuscularly.

CASE 3.—Patient, V. W., a 58-year-old woman, was admitted July 12, 1945, to a hospital after an illness of one month's duration. The symptoms were sore throat, fever, and persistent headache. The patient had received 300,000 units of penicillin without improvement. On July 15, 1945, the patient became lethargic and developed signs of meningeal irritation, namely, rigid neck and positive Kernig and Brudzinski signs. There was also a slight left hemiparesis, and the left knee jerk was greater than the right. The lungs were clear, a short apical systolic heart murmur was heard, and the spleen was enlarged. Lumbar puncture yielded 20 c.c. of cloudy fluid from which pneumococcus type III was cultured. The patient was treated with large doses of penicillin intrathecally and intramuscularly and sulfadiazine

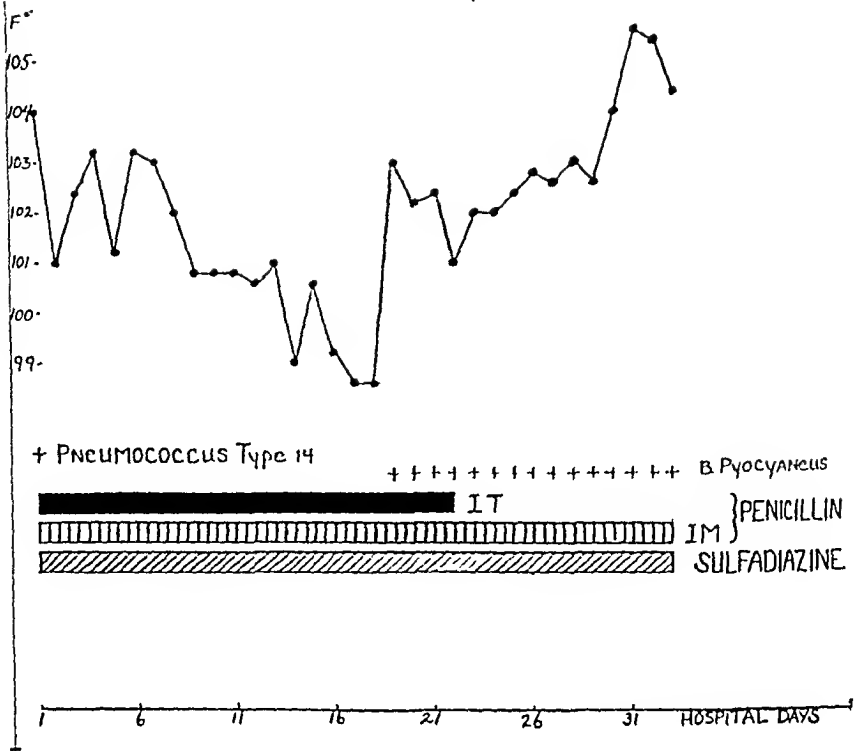


Fig. 2.—Case 2.

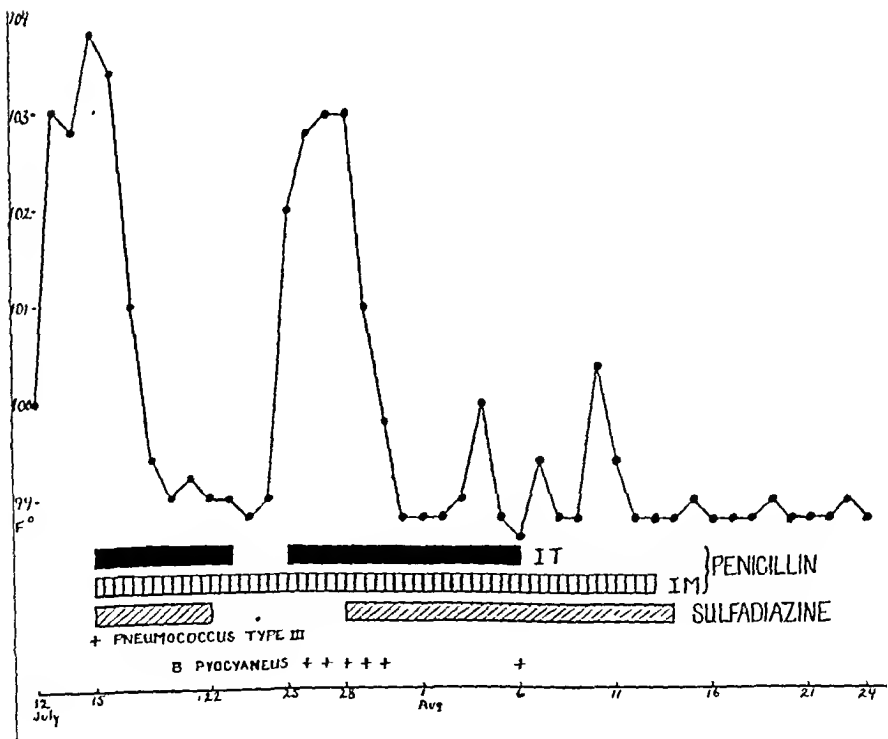


Fig. 3.—Case 3.

parenterally and orally. This treatment was continued for six days, after which the intrathecal penicillin and oral sulfadiazine were discontinued because of general improvement and sterile spinal fluid cultures.

Four days later the temperature rose to 102° F. (Fig. 3), and a cloudy spinal fluid was obtained from which *B. pyocyaneus* was cultured. This was confirmed by five subsequent positive cultures of the spinal fluid. For the succeeding 13 days oral sulfadiazine and intrathecal penicillin were given. Intrathecal penicillin therapy was then discontinued, but daily spinal drainage, intramuscular penicillin and sulfadiazine were continued until the temperature became normal. The patient left the hospital 48 days after admission, completely recovered.

CASE 4.—A. C., a 13-month-old infant, was hospitalized June 25, 1945, with a two-day history of fever and dehydration and convulsive tremors of the extremities for one day. Clinical signs of meningitis were present on admission, and type XVIII pneumococcus was cultured from the spinal fluid. No primary focus of infection could be found. Treatment consisted of large doses of sulfadiazine orally and penicillin intramuscularly for two weeks. For the first sixteen days of treatment the temperature fluctuated between 102 to 105° Fahrenheit. Intrathecal penicillin was given daily from July 10 to July 16 and from July 23 to August 3 because of persistent growth of pneumococcus from the spinal fluid. A middle ear infection became evident, and bilateral mastoidectomy was performed on July 31. From the right mastoid region *Staphylococcus aureus* was cultured, but no pneumococcus was found.

On Aug. 6, 1945, three days after the last intrathecal dose of penicillin, the patient's temperature rose to 102.6° F., and signs of meningeal irritation recurred. Cloudy spinal fluid was obtained from which *B. pyocyaneus* was cultured. No more intrathecal penicillin was used; however, the intramuscular dosage was continued for four more days, and oral sulfadiazine was continued in doses from 2.5 to 4.0 Gm. daily for the next nine days. The temperature gradually became normal and the patient completely recovered. *B. pyocyaneus* was recovered only on one occasion from the spinal fluid of this patient.

BACTERIOLOGIC INVESTIGATION

The day that the *B. pyocyaneus* was identified from the cultures of the spinal fluids of the two patients at Willard Parker Hospital, 20 vials of the penicillin that had been used for treatment on that ward were sent to the laboratory for study. These vials contained approximately 0.1 to 0.5 c.c. of clear penicillin solution diluted to 5,000 units per cubic centimeter. They were incubated at 37° C. without further dilution and showed no growth and were clear after forty-eight hours. However, after diluting the penicillin with 2 c.c. of beef heart infusion broth, by means of a syringe and needle inserted through the rubber stopper, *B. pyocyaneus* was recovered after twenty-four hours incubation at 37° C. or room temperature.

In order to check laboratory technique 16 additional vials, after use on that ward, were tested as follows: The aluminum band and rubber cap were removed, and 2 c.c. of broth were added from individual broth tubes by separate pipettes to each vial. All these vials of penicillin showed growth of *B. pyocyaneus* in twenty-four hours. The control broth tubes showed no growth after ninety-six hours of incubation at 37° Centigrade.

The method of preparing and administering the penicillin on the wards at our hospital was as follows: The penicillin was dissolved in 20 c.c. of saline drawn from a 500 c.c. "Sterisol flask." Individually wrapped syringes and needles, which were autoclaved, were used for the original dilution and for each

intrathecal dose. The rubber stopper was cleansed with alcohol before the needle was inserted. However, the intramuscular doses given at three-hour intervals were obtained by 2 c.c. syringes which were kept in a covered jar after having been boiled. Penicillin for both intramuscular and intrathecal doses for all patients receiving the drug were withdrawn from one vial until it was exhausted.

In order to determine the source of the *B. pyocyaneus* on the ward in which the infection occurred, penicillin from three fresh vials was cultured on the ward immediately after the diluent (saline) was added and also at the time of each (three- to four-hour intervals) intramuscular dose. Two-tenths cubic centimeter of penicillin from the syringe containing the intramuscular dose was added to 3 c.c. of broth before injection. The broth tubes were then incubated for ninety-six hours, and readings were made daily. Gram stains and subcultures into broth and on agar were made to identify the microorganisms found.

After ninety-six hours incubation no growth was observed in the cultures of the diluent (saline) and the penicillin immediately after dilution. However, *B. pyocyaneus* was found in all cultures of the penicillin which was drawn into 2 c.c. syringes for intramuscular injection.

At the same time the procedure outlined was also followed in the laboratory. One vial of penicillin was freshly diluted, and ten 0.2 c.c. samples at three-hour intervals were obtained by puncturing the disinfected rubber cap with a needle and a syringe. None of the cultures of the samples taken in the laboratory showed growth at the end of ninety-six hours of incubation.

In the laboratory the method of preparing and withdrawing the penicillin was the same as that on the ward, except that the 2 c.c. syringes used in the laboratory had been sterilized by dry heat in test tubes.

All the objects in the ward treatment room which could possibly be a source of contamination were cultured at the same time. The following results were obtained:

1. The jar in which the syringes were kept after boiling showed growth of *B. pyocyaneus*.*
2. Syringes (barrel and plunger) in the jar, ready for use, showed growth of *B. pyocyaneus*.
3. Syringes (barrel and plunger), immediately after boiling, showed no growth.

*The jars containing the syringes, the syringes, and the forceps used for taking them out were boiled in a fish kettle by the night nurses every twenty-four hours. The forceps and syringes were boiled also at other times if they came in contact with contaminated material. The jars were kept on the "dressing" table and were closed at all times, except when the syringes were being removed. The syringes were boiled unassembled, and the barrel and plunger were kept together by a rubber band during sterilization and kept in the jar. The sterilized forceps were kept in 0.4 per cent solution of lysol while not in use. There was, however, no way of checking whether during the wartime shortage of nurses and pressure of work this technique always had been carried out as outlined and whether the jars had actually been sterilized every night. It is probable that some of the jars had become contaminated through some breach of technique and that freshly sterilized syringes became in turn contaminated when placed in the jars.

The only other patient treated at the same time was a 6-year-old boy who was given penicillin intramuscularly. He had a rise in temperature at the same time as the other two patients (Cases 1 and 2). No local abscess appeared at any time, and his recovery was uneventful.

4. The sterilizer used for boiling the syringes showed no growth.

5. The box in which the penicillin was kept in the refrigerator as well as the caps on 2 vials in the box showed a few colonies of *Staphylococcus albus*.

6. Top and shelves and tray on the treatment table showed *Staph. albus* and *Aerobacter aerogenes*.

7. The window sill showed few *Staph. albus* and *A. aerogenes*.

8. Lysol and stock 70 per cent alcohol and the alcohol cotton wipes showed no growth.

From these studies on the ward in which the infection occurred, we were able to conclude that the syringes after being sterilized by boiling were contaminated by being kept in a jar harboring *B. pyocyaneus*. These syringes, when used to withdraw the intramuscular doses of penicillin, contaminated the drug.

After the source of contamination was traced in the ward in question, vials having some residual penicillin, the jars in which the syringes ready for use were kept, and the barrels and plungers from all the other wards in the hospital were cultured. Of the 21 vials tested, 3 vials showed growth of *B. pyocyaneus* and 4 of *Staph. albus* and of *A. aerogenes*. Two syringes showed growth of the aerobacter and two others *Staph. albus*.

A total of 61 vials of penicillin were cultured from all wards; of these, 41 showed growth of *B. pyocyaneus*, 39 from the ward where the accidental infections occurred. The vials of penicillin containing 5,000 units per cubic centimeter from which either the *B. pyocyaneus* or the *A. aerogenes* was obtained were clear. The vials that showed contamination were not from any particular lot number or from only one commercial biologic laboratory.

To insure absolute sterile conditions for the dilution and administration of penicillin, the following procedure was adopted at the Willard Parker Hospital:

1. Individual 20 c.c. ampules of saline are used to dilute the penicillin.

2. All needles and 2 c.c. syringes are assembled and placed in test tubes, and large syringes are individually wrapped and then all equipment sterilized in the autoclave.

3. Caps of the penicillin vials are washed with individual iodine and alcohol swabs before puncturing.

4. Individual vials of penicillin are used for each patient and are labeled with the name, date, and ward number.

DISCUSSION

Penicillin solutions used on the wards in three hospitals became contaminated with *B. pyocyaneus*. As the solutions remained clear they were considered sterile and were used therapeutically. The intramuscular injections produced no abscesses at the site of inoculation; the intrathecal injections, however, resulted in *B. pyocyaneus* meningitis in four patients with death in two cases.

Although penicillin in high concentrations may inhibit the active growth phase of either *B. pyocyaneus* or *A. acrogenes*, it has been established by Fleming⁷ and Abraham and associates⁸ that it is not bacteriocidal for these microorganisms. It is therefore important to maintain absolutely sterile technique in the preparation and administration of penicillin. This is especially important, since the penicillin shows no visual evidence of contamination and remains clear even at a concentration of 5,000 units per cubic centimeter and only becomes cloudy after it is diluted to contain less than 1,000 units per cubic centimeter.

SUMMARY

1. Secondary *B. pyocyaneus* meningitis following intrathecal penicillin treatment for pneumococcus meningitis was observed in four patients, two of whom died.

2. The source of the *B. pyocyaneus* was traced in two of the cases to penicillin solutions contaminated by syringes used to withdraw the drug for injection.

3. As penicillin solutions containing 5,000 units per cubic centimeter show no evidence of contamination, absolutely sterile technique in the preparation and administration of penicillin must be observed.

We wish to extend our thanks to Dr. Vera B. Dolgopel and Dr. Jerome L. Kohn, for their aid in preparation of this paper, and to Miss Ruth Gosling, for her assistance in the bacteriologic diagnosis.

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THE TWO-DOSE DEXTROSE TOLERANCE TEST IN THE DIAGNOSIS OF DIABETES MELLITUS

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THE standard single dose dextrose tolerance test, first used by Jacobson,¹ has not proved entirely satisfactory for the diagnosis of diabetes mellitus. The procedure is not only time-consuming and discomforting but also lacking in sensitivity and specificity.²⁻⁴ Moreover, the results of the test are influenced significantly by the composition of the antecedent diet.⁵⁻⁹

To vitiate the deficiencies in the standard test, several modifications have been proposed. One of these, in which two doses of dextrose are given one to two hours apart, was suggested by Staub¹⁰ and Traugott.¹¹ In the hands of Malmros¹² and Julesz¹³ this procedure seemed none too satisfactory, and so fell into disuse.

The two-dose test was revived and further modified by Exton and Rose,¹⁴ who shortened the interval between the first and second dose of dextrose to one-half hour and who abbreviated the duration of the procedure to one hour.

The two-dose one-hour test subsequently has gained considerable popularity. It has been used in the Out-Patient Diabetes Clinic of Strong Memorial Hospital* since 1938. We propose herein to summarize our experience with the test, which in clinic practice has been timesaving, economical, and satisfactory for the diagnosis of diabetes mellitus. Of special interest are the data concerning the value of an additional sugar determination made two hours from the beginning of the test.

Technique of the Test.—All individuals tested were ambulant patients in the Out-Patient Department of Strong Memorial Hospital. Each was instructed to take neither food nor insulin from the time of the evening meal until the test had been completed the following morning. No attempt was made to control the diet preceding the test.

The sugar solution was prepared by dissolving 100 Gm. of dextrose in 650 c.c. of water. The resulting 15 per cent solution was divided into two equal portions.

After the patient had voided, a specimen of venous blood was withdrawn and the first portion of the dextrose solution given. Two minutes were allowed for its ingestion. Thirty minutes later, blood and urine specimens were obtained and the second portion of the dextrose solution given. Third and fourth specimens of blood and urine were obtained one hour and two hours, respectively, after the first dose of dextrose solution had been given.

The Physiologic Basis of the Two-Dose Test.—The two-dose dextrose tolerance test is based upon sound physiologic principles. The first of these, known as Allen's¹⁵ law of paradoxie dextrose utilization, is the fact that the more dextrose ingested by a normal individual the more is utilized. The patient with diabetes mellitus, on the other hand, limited in his ability to cope with carbohydrates, develops hyperglycemia and usually glycosuria after the ingestion

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of sizable amounts of dextrose. The second principle, the Hamman-Hirschman¹⁵ phenomenon, is that when dextrose is given to nondiabetic persons in two increments the hyperglycemia which follows the second dose is less than that produced by the first. The diabetic patient, less adept in removing excess carbohydrate from the blood, responds with increased hyperglycemia to repeated doses of dextrose.

Advantages and Disadvantages of the Two-Dose Test.—The principal advantages of the two-dose test are its brevity, simplicity, and economy. The shorter duration of the test diminishes the effect of factors such as hunger, fatigue, and impatience. Fewer venipunctures and fewer chemical determinations are necessary than with the standard test.

There is some reason to believe that the composition of the antecedent diet distorts the results of the two-dose test less than those of the standard one-dose procedure. Presumably, this is because the first increment of dextrose acts as a buffer against whatever depletion of liver glycogen may have occurred before the test is performed.

The results obtained by groups of investigators studying the influence of the antecedent diet upon the two-dose one-hour test are at variance. Exton and Rose state that moderate variations in the diet do not influence the test. Sweeney, Muirhead, and Allday,¹⁷ drastically altering the composition of their subjects' diets, found effects they considered significant, and Langner and Fies¹⁸ state that "moderate as well as extreme manipulation of the diet is capable of altering the Exton-Rose glucose tolerance from normal to the diabetic type." Wayburn and Gray,¹⁹ however, found the two-dose test "relatively free from the influence of fairly marked changes in the preceding diet and therefore superior to the one-dose test when the preceding diet is not known."

A disadvantage of the test noted by Young²⁰ is that when dextrose absorption is unusually rapid, the two-dose test gives an erroneous picture of the true status of the patient's carbohydrate tolerance. In our experience these individuals may be identified by prolongation of the two-dose test to include a two-hour specimen.

Another disadvantage of the test, although one seldom encountered, is that nervousness may delay passage of the ingested dextrose from the stomach into the intestine. Gastric motility varies considerably from subject to subject,^{21, 22} and, in a few, delay in the emptying time of the stomach may retard the hyperglycemic response, a factor which merits consideration because of the brief time interval involved.

Comparison of the Two-Dose Test With the Standard One-Dose Procedure.—No extensive comparison between the standard one-dose test and the two-dose procedure is available. Kelly, Beardwood, and Fowler²³ found the two tests in close agreement and cite two cases out of fifty in which the two-dose test was found to be superior. Brown²⁴ also has compared the two procedures and found them equally accurate. Langner and Dewees,²⁵ who studied 160 patients, believe that the conventional one-dose test is the more specific of the two, although they consider the Exton-Rose test somewhat more sensitive.

Interpretation of the Two-Dose Test.—The most cogent reason why the two-dose procedure has not displaced the standard dextrose tolerance is that there has not been complete agreement as to the interpretation of the results.

The criteria of Exton and Rose²⁶ and those of Gould, Altshuler, and Mellen²⁷ are at variance. The most extensive study of the test is that of Matthews, Magath, and Berkson,²⁸ and most subsequent workers have agreed with their interpretation.

TABLE I. PROPOSED CRITERIA FOR INTERPRETATION OF EXTON-ROSE TEST

-
1. Exton and Rose²⁶
 - A. Normal tolerance curve:
 - (1) Normal fasting blood sugar
 - (2) Rise in blood sugar during first half hour not to exceed 75 mg. per 100 c.c.
 - (3) Rise in blood sugar during second half hour not to exceed 5 mg. per 100 c.c.
 - B. Diabetic tolerance curve:
 - (1) A more or less steep curve of not less than 10 mg. of blood sugar following the second dose of dextrose.
 - (2) Relation of blood and urine sugar values to the severity of the disease.
 2. Gould, Altshuler, and Mellen²⁷
 - A. Normal tolerance curve:
 - (1) Fasting blood sugar less than 120 mg. per 100 c.c.
 - (2) The one-half hour level is less than 50 mg. per 100 c.c. above the fasting value.
 - (3) The level of the blood sugar at one hour is less than 30 mg. above the one-half hour level.
 - B. Diabetic tolerance curve:
 - (1) Fasting blood sugar of 120 mg. or more.
 - (2) One-half hour level of 50 mg. or more above the fasting.
 - (3) One hour level of 30 mg. or more above the one-half hour level.
 - (4) Two of these three criteria necessary for diagnosis.
 3. Matthews, Magath, and Berkson²⁸
 - A. All individuals with blood sugar level at one hour of less than 158 mg. per 100 c.c. considered nondiabetic, and all over 180 mg. per 100 c.c. are considered diabetic. Those between 158 and 180 mg. are nondiagnostic.
-

With the criterion of Matthews, Magath, and Berkson, we also agree. In our experience the one-hour blood sugar value is the most reliable indication of the presence or absence of diabetes mellitus. We believe, however, that implicit reliance on a single chemical determination may be misleading. No test of physiologic function as complex as is the regulation of the blood sugar can be expected to be definitive. That this is true of the two-dose dextrose tolerance test is apparent from the material upon which this study is based.

Each case should be viewed in its entirety, and special consideration should be given to the following: the existence of diabetes in members of the patient's family, the presence of obesity or of diseases of organs concerned with carbohydrate metabolism (liver, hypophysis, thyroid), and the effect of infections upon the patient's carbohydrate tolerance.

ANALYSIS OF CASES

In the present study a statistical analysis has been made of the results of the two-dose test performed on twenty-six normal persons, eighty-two in whom a diagnosis of diabetes mellitus was considered to have been established, seven with renal glycosuria, and six subjects whose dextrose tolerance curves are considered borderline or doubtful.

The diagnosis of diabetes mellitus was established by considering the following: the symptoms of which the patient complained, a history of the

TABLE II. AVERAGE BLOOD SUGAR READINGS

	NUMBER OF CASES	FASTING		
		MEAN	RANGE	± OF MEAN
Nondiabetic				
Normal	26	77.4	61 to 94	8.5
Renal glycosuria	7	83.1	72 to 91	6.9
Diabetic				
No insulin	49	138.2	75 to 331	48.8
Insulin at onset only	11	153.5	96 to 233	43.6
Insulin treatment	22	179.7	76 to 315	66.8
Total diabetic	82	151.4	75 to 334	55.8

disease in other members of the family, the absence of other conditions associated with hyperglycemia, the degree of glycosuria, the patient's reaction to infections, and the results of the two-dose test.

Of the eighty-two patients with diabetes mellitus: twenty-two required insulin daily; forty-nine were regulated satisfactorily without insulin; and

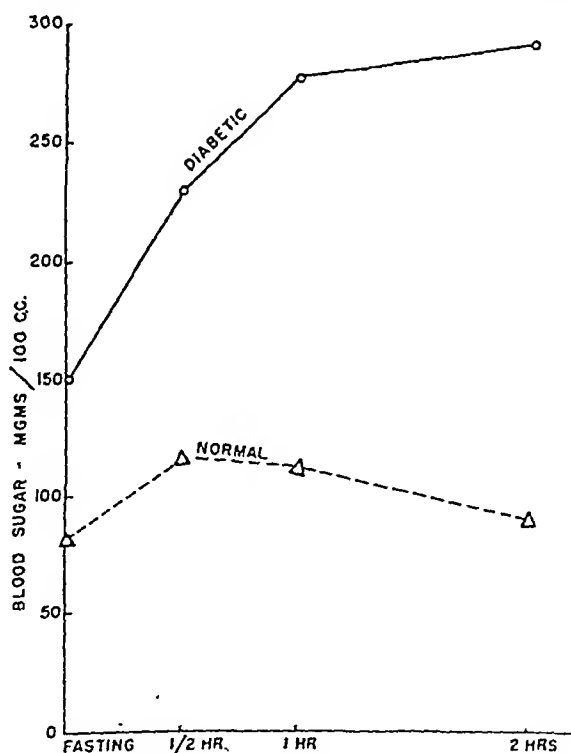


Fig. 1.—Average blood sugar readings with the two-dose test.

eleven were given insulin at the onset but subsequently were able to dispense with the injections as their condition improved from weight reduction, dietary regulation, and control of infections.

No attempt was made to subclassify the group of insulin-treated diabetic patients according to the amount of insulin required. Nor did there seem any

TWO-DOSE DEXTROSE TOLERANCE TEST

ONE-HALF HOUR			ONE HOUR			TWO HOURS		
MEAN	RANGE	δ OF MEAN	MEAN	RANGE	δ OF MEAN	MEAN	RANGE	δ OF MEAN
5.6	84 to 135	14.4	113.9	74 to 150	21.5	91.9	56 to 125	19.9
5.8	106 to 124	9.8	112.2	91 to 127	14.6	101.0	79 to 125	13.8
22.4	99 to 475	67.7	266.8	180 to 500	63.3	268.8	135 to 500	87.1
12.9	96 to 333	72.9	283.2	222 to 377	77.4	291.4	182 to 421	109.9
30.6	118 to 385	70.6	307.1	170 to 444	77.5	348.4	136 to 500	102.0
31.4	96 to 475	70.5	279.8	170 to 500	70.5	295.6	135 to 500	99.7

special virtue in trying to evaluate the "severity" of diabetes from the results of the dextrose tolerance test alone.

The results of the tests, expressed as average readings for each group, are shown in Table II and represented graphically in Fig. 1.

TABLE III. ANALYSIS TO DETERMINE STATISTICAL VALIDITY OF CRITERIA FOR INTERPRETATION OF TWO-DOSE TEST

	NORMAL		DIABETIC		δ OF DIFFER- ENCE OF MEANS	$\bar{x} =$ $M_1 - M_2$	$\frac{\bar{x}}{\delta}$
	MEAN	δ OF MEAN	MEAN	δ OF MEAN			
Fasting							
Height of fasting blood sugar	77.4	8.5	151.4	55.8	55.6	74.0	1.33
One-half hour							
Height of one-half hour blood sugar	115.6	14.4	231.4	70.5	71.8	115.8	1.61
Milligrams change; fast- ing, one-half hour	38.2	13.4	80.0	43.1	45.0	41.8	0.93
Percentage change; fast- ing, one-half hour	49.3	19.2	52.8	41.7	45.9	3.5	0.08
One hour							
Height of one hour blood sugar	113.9	21.5	279.8	70.5	73.7	165.9	2.25
Milligrams change; one- half to one hour	-1.7	19.5	48.4	65.1	67.9	50.1	1.36
Percentage change; one- half to one hour	-1.5	17.0	20.9	21.4	27.3	22.4	0.82
Milligrams change; fast- ing, one hour	36.5	19.5	128.4	51.8	55.4	91.9	1.66
Percentage change; fast- ing, one hour	47.2	25.6	84.8	69.2	73.7	37.6	0.51
Two hours							
Height of two hours blood sugar	91.9	19.9	295.6	99.6	102.0	203.7	2.00
Milligrams change: one hour to two hours	-22.0	16.6	15.8	49.0	51.7	37.8	0.73
Percentage change: one hour to two hours	-19.3	14.3	5.6	17.4	22.6	24.9	1.10
Milligrams change: one- half hour to two hours	-23.7	20.6	64.2	58.5	62.1	87.9	1.42
Percentage change: one- half hour to two hours	-20.5	18.7	27.7	28.2	33.9	48.2	1.42
Milligrams change; fast ing, two hours	14.5	16.5	144.2	123.2	124.4	129.7	1.04
Percentage change; fast- ing, two hours	18.7	22.2	95.2	51.8	75.2	76.5	0.98

Consideration of the mean values alone indicates that in the average case, the two-dose procedure readily separates the diabetic patient from the non-diabetic person. Standard deviations of the mean values, however, tend to be large, especially in the diabetic groups. Further statistical analysis therefore was made in an attempt to determine which criteria are most reliable. The results of this analysis are summarized in Table III.

The data of Table III indicate that the height of the one-hour blood sugar reading is the most reliable criterion. The height of the two-hour blood sugar appears to be the next most valid differential point. The x/δ values of the other calculations are below 2.0, and therefore cannot be considered statistically significant.

From this analysis, we believe the data in Table IV to represent the most important considerations in attempting to differentiate the normal from the diabetic by the two-dose dextrose tolerance test.

TABLE IV. CRITERIA FOR DIFFERENTIATION OF NORMAL PERSONS FROM PATIENTS WITH DIABETES MELLITUS

	BLOOD SUGAR READINGS		
	NORMAL	DOUBTFUL	DIABETIC
One hour	Below 150	150 to 170	Above 170
Two hours	Below 125	125 to 135	Above 135

TABLE V. CORRECTNESS OF PROPOSED DIAGNOSTIC CRITERIA

	TOTAL NUM- BER OF CASES*	EXTON AND ROSE		GOULD AND ASSOCIATES		MATTHEWS AND ASSOCIATES		CONNER AND REYNOLDS	
		NUM- BER	PER- CENT- AGE	NUM- BER	PER- CENT- AGE	NUM- BER	PER- CENT- AGE	NUM- BER	PER- CENT- AGE
Normal	29	20	69	21	72	27	93	29	100
Diabetic	85	78	92	77	91	82	96	84	99

*Includes six borderline cases summarized in Table VI.

All of the cases, both normal and diabetic, summarized in Table II meet both of the criteria set forth previously. The number of correct diagnoses which would have been made had the criteria of others alone been relied upon is shown in Table V.

ANALYSIS OF BORDERLINE CASES

There remain nine cases in which the diagnosis of diabetes mellitus could neither be readily established nor definitely excluded. These cases are detailed in Table VI.

In Cases 1 to 3, there was clinical evidence of hepatic or pituitary disease.

Cases 4 and 5 are considered diabetes mellitus, although the one-hour reading leaves some doubt as to this diagnosis. The low blood sugar at one hour may represent delay in gastric emptying time. Cases 6 to 8 probably represent instances of unusually rapid dextrose absorption by nondiabetic persons, as suggested by Young. Technical error in the blood sugar determinations cannot be ruled out. Case 9 is unique in our experience. This patient, when studied in the Out-Patient Department, showed no evidence of diabetes, although two years previously he was said to have had glycosuria during

TABLE VI. SUMMARY OF BORDERLINE CASES

CASE	PATIENT	CLINICAL DIAGNOSIS	TWO-DOSE TEST	DIAGNOSIS BY CRITERIA OF:		
				EXTON AND ROSE	GOULD AND ASSOCIATES	MATTHEWS AND ASSOCIATES
1	V. L.	Cirrhosis of liver	95-143-182-118	Diabetes	Indetermi- nate	Diabetes
2	C. J.	Cirrhosis of liver	87-133-163 65-115-160-156	Diabetes	Normal	Indetermi- nate
3	R. M.	Pituitary basophilism	80-161-179-145	Diabetes	Normal	Indetermi- nate
4	B. S.	Diabetes	84-118-164-160	Diabetes	Indetermi- nate	Indetermi- nate
5	A. V.	Diabetes	74-111-140-160 86-95-168-165	Diabetes	Normal Indetermi- nate	Normal Indetermi- nate
6	N. P.	Rapid absorption	100-208-135-90	Normal	Indetermi- nate	Normal
7	E. J.	Rapid absorption	93-160-184-83	Diabetes	Indetermi- nate	Diabetes
8	L. B.	Rapid absorption	87-139-175-118	Diabetes	Diabetes	Indetermi- nate
9	P. M.	Diabetes	82-111-127	Diabetes	Normal	Normal

hospitalization for drainage of a palmar abscess. Six months later he was admitted to the hospital with pyelonephritis and in the presence of infection again became frankly diabetic with hyperglycemia and glycosuria, requiring the use of insulin.

DISCUSSION

As with other laboratory procedures, the two-dose dextrose tolerance test should be used only to supplement other clinical considerations in attempting to differentiate the diabetic from the nondiabetic patient.

Properly interpreted, the test is a valuable procedure for making this differentiation. Greatest reliance should be placed upon the height of the blood sugar at one hour. However, it is distinctly helpful to see the complete curve and to correlate the readings with other factors. The one-half hour reading contributes little to the differentiation, but the two-hour reading has proved of great value in borderline cases. The results of the test should be interpreted with reserve in the presence of diseases of the liver, thyroid, or pituitary gland.

CONCLUSIONS

1. The two-dose dextrose tolerance test is based on sound physiologic principles and is sufficiently sensitive and specific to make it a valuable procedure for the diagnosis of diabetes mellitus in clinic and office practice.

2. The height of the one-hour blood sugar is the most reliable criterion for differentiating the normal person from the diabetic patient.

3. Determination of the blood sugar at one-half hour contributes little to the differentiation and may well be deleted from the procedure. The two-hour reading, however, is reliable and should be included in all doubtful cases.

4. In a few cases no definitive diagnosis can be established from the results of the dextrose tolerance test alone. These cases should be viewed in their entirety, and special consideration should be accorded the clinical picture.

5. When the one-hour blood sugar reading is within the diabetic range and the two-hour reading normal, excessively rapid dextrose absorption should

be suspected. When the one-hour blood sugar reading is normal and the two-hour specimen indicative of diabetes, delayed gastric emptying is to be considered.

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A DETAILED REPORT ON THE WEIGHTS AND WEIGHT LOSSES OF TWENTY-FOUR MEN IN SANTO TOMAS INTERNMENT CAMP

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ALTHOUGH there have been a number of reports on the nutrition in various prison and internment camps of World War II, there has been to my knowledge no detailed report on a group by one who knew the individuals and their condition intimately. It was therefore considered to be of value to report the following data.

In Table I are presented the weights of twenty-four men civilian internees of Santo Tomas Internment Camp in Manila, P. I., thirty-six months after their internment and two weeks before liberation on Feb. 3, 1945. The classification of body types was made by the author who had lived in either the same or adjoining rooms with these men for eighteen months or more. The ideal weights were taken from tables compiled by the Statistical Bureau of the Metropolitan Life Insurance Company (Wohl¹). This particular group of men cannot be said to be entirely typical of the camp population, as they were the more healthy individuals remaining from two rooms after the other members not so fortunate had been transferred to quarters considered to be more suitable for those in a poor state of health. In fact this group, in general, had been able to obtain considerable amounts of supplementary food in addition to the camp ration. For example the author had been able to add about 3 ounces of canned meat daily to his diet and about 1 ounce of salad oil during the last five months. This, in addition to consuming large amounts of peanut butter as long as it was available (until about May, 1944) and gorging himself with rice as long as it was available (until about Feb. 1, 1944), allowed him to keep his weight at 93 per cent of the ideal. However, in spite of this, his blood pressure was 90 mm. Hg instead of a normal 127 mm. Hg; pulse, 48 to 50 instead of a normal 70; red blood cells, 3.25 millions per cubic millimeter instead of 5.5 millions per cubic millimeter; and the hemoglobin about 55 per cent. At this time a blood pressure of 100 mm. Hg and a red blood cell count of 4 millions per cubic millimeter or over was considered exceptional in the camp population.

Perhaps it should be added that the low red blood cell and hemoglobin values of the author were no doubt in part due to three donations for transfusion of about 500 c.c. each given in the latter part of 1943 which dropped the red blood cells to 3.5 millions per cubic millimeter. Early in 1944 a treatment with injectible liver preparations plus 70 Gm. of peanut protein daily raised this to 4.25 millions per cubic millimeter in six weeks. Several months

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TABLE I. DETAILED DATA ON WEIGHTS AND WEIGHT LOSSES OF TWENTY-FOUR MEN INTERNEES OF SANTO TOMAS INTERNMENT CAMP, MANILA,
P. I., AFTER THIRTY-SIX MONTHS OF INTERNMENT AND TWO WEEKS BEFORE LIBERATION

NO.	SUBJECT	AGE (YEARS)	FRAME TYPE†	HEIGHT (INCHES)	WEIGHT					FINAL AS PER CENT OF IDEAL	
					PREWAR (POUNDS)	IDEAL (POUNDS)	FINAL (POUNDS)	LOSSES (POUNDS)	FINAL AS PER CENT OF		
									PREWAR		IDEAL
1	P. K.*	18	L	67	--	135	93	--	--	69	--
2	L. C.	33	M-H	69	160	160	130	30	81	81	100
3	B. H. B.	34	M-H	68	165	156	145	20	88	93	106
4	J. S.	34	H	67	185	155	125	60	68	81	119
5	P. E. M.	35	L	68	135	140	113	23	84	81	96
6	R. H.	35	M	71	170	163	123	47	72	75	104
7	R. B. D.	36	M-H	72	178	173	145	33	81	84	103
8	L. C.	36	L	72	145	158	135	10	93	86	92
9	A. E. L.	36	H	72½	190	185	150	40	79	81	103
10	D. C. B.	37	L	68	148	142	110	38	74	77	104
11	W. D.	38	M	71	162	162	114	48	70	70	100
12	G. M.	38	L	70½	140	155	110	30	79	71	90
13	R. G.†	39	L	69	170	146	98	72	58	67	117
14	L. B.	39	L-L	73	153	137	122	31	80	78	98
15	D. C.	40	M-L	68	148	147	125	23	84	85	101
16	B. P.	40	M	68	190	150	127	63	67	85	127
17	S. C.	43	L	70	157	150	114	43	73	76	105
18	F. V. K.	47	M	68½	185	156	137	48	74	88	119
19	M.	55	M	67	160	146	114	46	71	78	110
20	R.†	58	L	70	157	150	120	37	76	80	105
21	N. G.	60	L	70	186	150	122	64	65	81	124
22	H.	65	L	68	178	142	115	63	65	81	125
23	B. H. B.	65	H-H	66½	187	160	141	46	75	88	117
24	S. K.†	74	M	70	178	158	127	51	71	80	113
Averages					166	155	124	42	75	80	108

*Excluded from averages.

†L, M, and H refer to light, medium, and heavy frame types, respectively.

‡Slight edema in lower extremities.

later, when the count had again fallen off considerably, a second series of liver injections without the supplementary protein (no longer available) had no effect on the red blood cell count.

Keys and co-workers,² in experiments with thirty-four men, found that edema had developed in nearly all cases in a six months' starvation period during which the subjects lost 25 per cent of their weight and were on a protein level of 49 Gm. derived from vegetable sources. It therefore seems rather remarkable that so few of our group showed edema at this time, for as is shown in Table I we had lost on an average of 25 per cent in weight although over a longer period of time. As indicated in Table I only Subjects 13, 20, and 24 showed definite edema at this time. In the two weeks which ensued after these data were collected and before liberation, Subjects 1, 5, 10, 21, and 22 also showed traces of edema. Our calorie level from the camp ration had been less than 1,000 for over five months and the protein level at 20 to 30 Gm. obtained chiefly from rice and corn with a few beans, sweet potatoes, and negligible amounts of meat. Even the author, whose diet was supplemented by about 3 ounces of meat daily, received only 40 to 50 Gm. of protein daily. It seems possible that we had gradually become adapted to a low level of protein and general metabolism, as the blood pressure and pulse rates would suggest.

TABLE II.* AVERAGE WEIGHTS OF VARIOUS AGE GROUPS AT SANTO TOMAS INTERNMENT CAMP, MANILA, P. I., THIRTY-SIX MONTHS AFTER INTERNMENT AND TWO WEEKS BEFORE LIBERATION

SUBJECTS	AGE	NUMBER OF PERSONS	AVERAGE WEIGHTS			
			PREWAR (POUNDS)	36 MONTHS LATER (POUNDS)	LOSSES (POUNDS)	PER CENT OF PREWAR
Men	19 to 40	508	166	124	42	75
Men	41 to 60	555	175	122	53	70
Men	Over 60	433	177	119	58	67
Camp totals		1,506	172	121	51	70
Women	19 to 40	667	125	101	24	81
Women	41 to 60	448	135	100	35	74
Women	Over 60	119	149	96	53	64
Camp totals		1,232	132	100	32	76

*Official Records of Santo Tomas Internment Camp. Unpublished.

It is interesting to note that our camp average of 121 pounds (Table II) for men was from 10 to 15 pounds below that of 4,618 prisoners of war freed in the Far East and reported by the military authorities.³

Table II also bears out the general observation made by the author that the women fared considerably better than the men. Their weight at this time was 76 per cent of normal as against 70 per cent for the men. This was probably largely due to the fact that they received the same food rations as the men in spite of the fact that their requirements were considerably less. It is also noticeable (Table II) that the older and more obese individuals lost more weight and reached a final lower weight than the younger people. This was probably due to several factors. In the first place, the younger people worked more actively, especially around the kitchen, and thus received more food in

many cases. The younger ones were more vigorous and more able to produce extra food in gardens of their own or to do work for other individuals in camp and thus obtain either directly or indirectly more food. The younger people also were not so particular about their food and regardless of former food habits ate rice, unappetizing stew, or anything else that could be had, while many of the older people, in the earlier days when considerable amounts of food were thrown away daily, refused to eat heartily and thus started to decline long before it was necessary.

The unusual case of an extremely obese individual (B. C., Negro) who entered camp weighing 365 pounds and dropped to 148 pounds, a loss of 217 pounds or 59.4 per cent of his total weight, is also noted.⁴

It might be of interest to point out that, in general, this group of men had lived for extended periods in the tropics. Several had been born in the Islands and except for visits and education abroad had spent their lives there. Two others, Subjects 24 and 25, had spent approximately forty years each in the Islands. Many others had lived there from five to ten years. The relative condition of these individuals suggests that the old idea of the unhealthy climate of the tropics is largely a myth if one follows reasonable rules of health and sanitation.

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THE COMPARATIVE EFFICIENCY OF VARIOUS LIVER FUNCTION TESTS IN DETECTING HEPATIC DAMAGE PRODUCED IN DOGS BY XYLIDINE

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STUDIES concerning the comparative values of different liver function tests on human beings,¹⁻³ cats and rabbits,⁴ and dogs,⁵ with references to the original methods, have been published during the past few years. Hepatic damage in the cats and rabbits cited was induced by daily ingestion of selenium (from 0.1 to 1.0 mg. per kilogram) and, in the dogs, by small doses of carbon tetrachloride several times a week.

During the course of an investigation carried out in this laboratory with various species of animals, it was found that jaundice and fatty livers were produced in cats and dogs by a moderately low concentration of xylidine, an organic amine having the formula $C_6H_5(CH_2)_2NH_2$. This report is concerned with the evaluation of various liver function tests in reference to their efficiency and practicability in permitting early diagnosis in cases of experimentally induced liver damage in dogs.

EXPERIMENTAL METHODS

Thirteen adult female dogs weighing from 5.1 to 8.0 kilograms were used as experimental animals. They had been dewormed, kept under observation for at least six weeks prior to the experimental period, and had shown no signs of distemper or other respiratory infection.

Seven dogs (Group I), of which three were used as controls, were fed a diet (Diet A) consisting of 46 per cent horsemeat, 46 per cent kibbled biscuits, 4 per cent Sure's salt mixture,⁶ and 4 per cent dried brewers' yeast. For comparative purposes, six dogs (Group II), of which three were used as controls, were fed a diet (Diet B) consisting of 30 per cent technical casein, 27 per cent crackermeal, 23 per cent sugar, 9 per cent Crisco, 4 per cent Sure's salt mixture, 4 per cent dried brewers' yeast, and 3 per cent cod-liver oil. The dogs in each group received 20 Gm. per kilogram of the assigned diets daily and showed a weight gain of approximately 0.1 kilogram weekly as long as they ate and retained the food. As will be noted, the results of the liver function tests and microscopic examination of the livers did not indicate any difference with respect to these diets.

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Hepatic damage was produced by exposing the dogs to a vapor-air mixture of xylydine of a high degree of purity. The inhalation exposures were of approximately six hours' duration daily, Monday through Friday. The average calculated concentration of xylydine in the chambers was 0.42 mg. per liter (85 p. p. m.), while the average concentration as determined by ultraviolet absorption was 0.22 mg. per liter (45 p. p. m.). Vomiting, loss of appetite, weakness, labored respiration, and jaundice were the usual symptoms observed. Weekly hematologic examinations revealed no marked changes. The survival time for the majority of the exposed dogs was approximately four or five weeks. Death occurred within a week after definite signs of jaundice were noted (jaundice occurring about one or two weeks after loss of appetite). On refusal of the dogs to eat the diet, forced feeding was instituted. Only one dog in Group II was able to retain the food. No signs of jaundice were noted even after fifteen weeks of exposure, when it was sacrificed and the liver found to be grossly nodular. Post-mortem examination of all the animals which died within five weeks showed marked jaundice around the limbs as well as the abdominal viscera, and the livers appeared pale and fatty.

In general, the methods of Smith and associates⁶ for determining hepatic damage in cats and rabbits were used for the bromsulfalein, rose bengal, and bilirubin retention tests. In all these experiments, 5 mg. of the dye (or bilirubin) per kilogram body weight were injected intravenously. Examinations of the dye in the plasma were made at five- and thirty-minute intervals for the bromsulfalein and rose bengal retention tests and at 5- and 120-minute intervals for the bilirubin retention test. All the estimations were made with the Fisher spectrophotometer using appropriate filters and control plasma solutions. The amount of intravenously injected substance per cubic centimeter of plasma obtained at the different time intervals was read from a calibration curve. The per cent retention of the dye was expressed in terms of the amount found at thirty minutes (the per cent retention of bilirubin being based on the amount found at the end of 120 minutes) as compared to that found at five minutes. Although some of the injected dye or bilirubin is removed from the blood stream within the five-minute period, the per cent retention determined by the foregoing method tends to minimize the effect of variables, such as the total blood volume compared to the body weight, the hematocrit values, or possible inaccuracies in the administration of the dye or bilirubin.

The method of Bodansky⁹ was followed in determining the serum phosphatase activity. Urobilinogen in the twenty-four hour urine specimens was determined by the method of Sparkman¹⁰ following treatment of a sample of urine with freshly prepared ferrous hydroxide to reduce any urobilin as suggested by Schwartz and co-workers.¹¹ Values for ieterus index were obtained by the visual comparison of saline-diluted plasma with potassium dichromate standards as outlined by Kolmer and Boerner.¹² The prothrombin clotting time was determined in plasma by the method of Quick¹³ using freshly prepared thromboplastin made from rabbit brain. The prothrombin time ratio was expressed as the clotting time of the plasma of the exposed as compared to that of

the control animals. The direct and indirect van den Bergh tests were performed according to standard procedures.¹² The albumin-globulin ratio and fibrinogen estimations were carried out as outlined by Reiner.¹⁴ Preliminary work with the cephalin-cholesterol test confirmed the observation of Hanger¹⁵

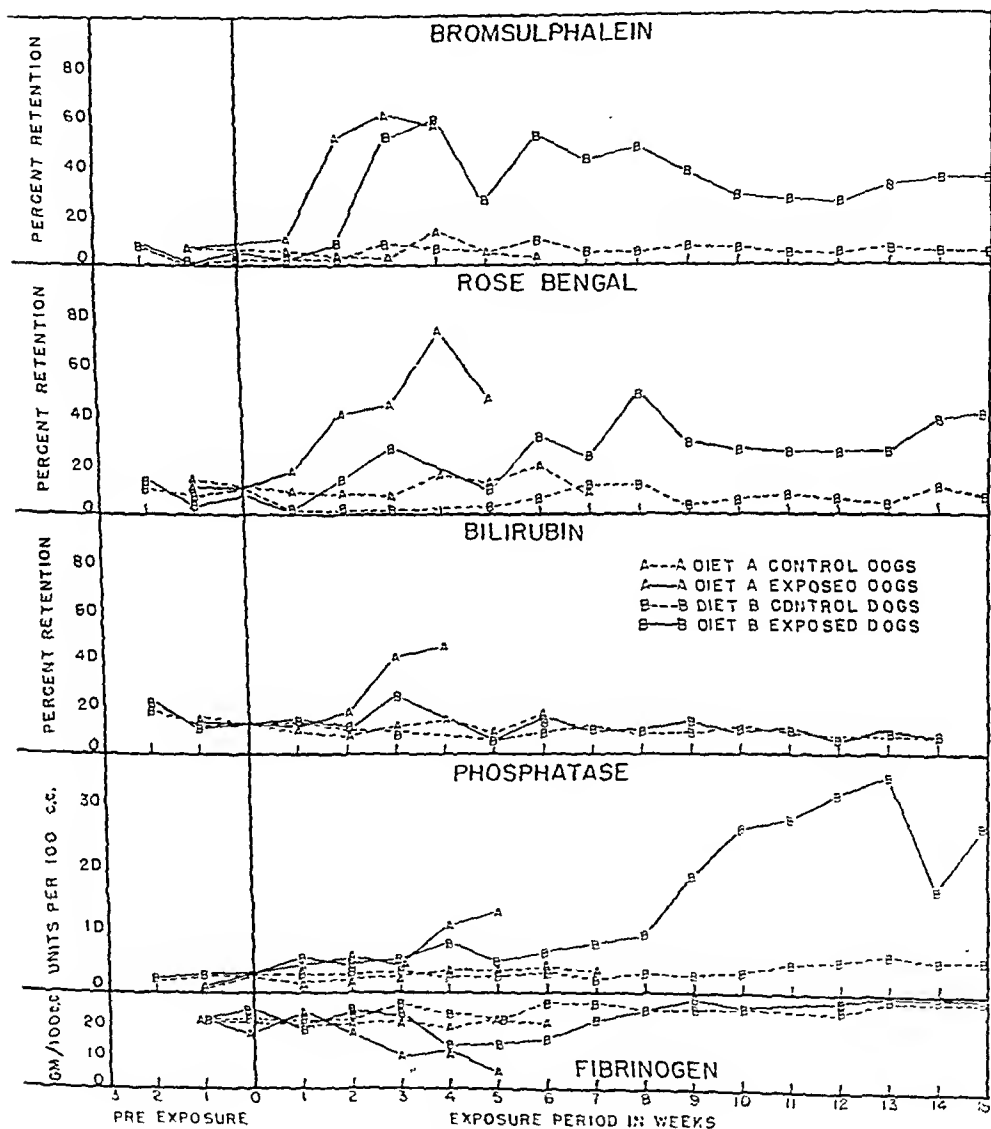


Fig. 1.—Liver function tests.

that marked flocculation occurred in the serum of normal dogs. For laboratory animals, this test has been reported to be of value only in monkeys.¹⁶

The various tests were performed at least sixteen hours after the previous day's exposure and feeding. All tests previously described were performed once a week, except the icterus index, prothrombin time ratio, and indirect van

den Bergh reactions which were determined twice weekly immediately after exposure. The urinalyses were done on twenty-four hour specimens collected each morning, Tuesday through Saturday.

Microscopic examination of the liver was done after fixation in a 4 per cent formaldehyde solution buffered to pH 7. Paraffin sections were routinely stained

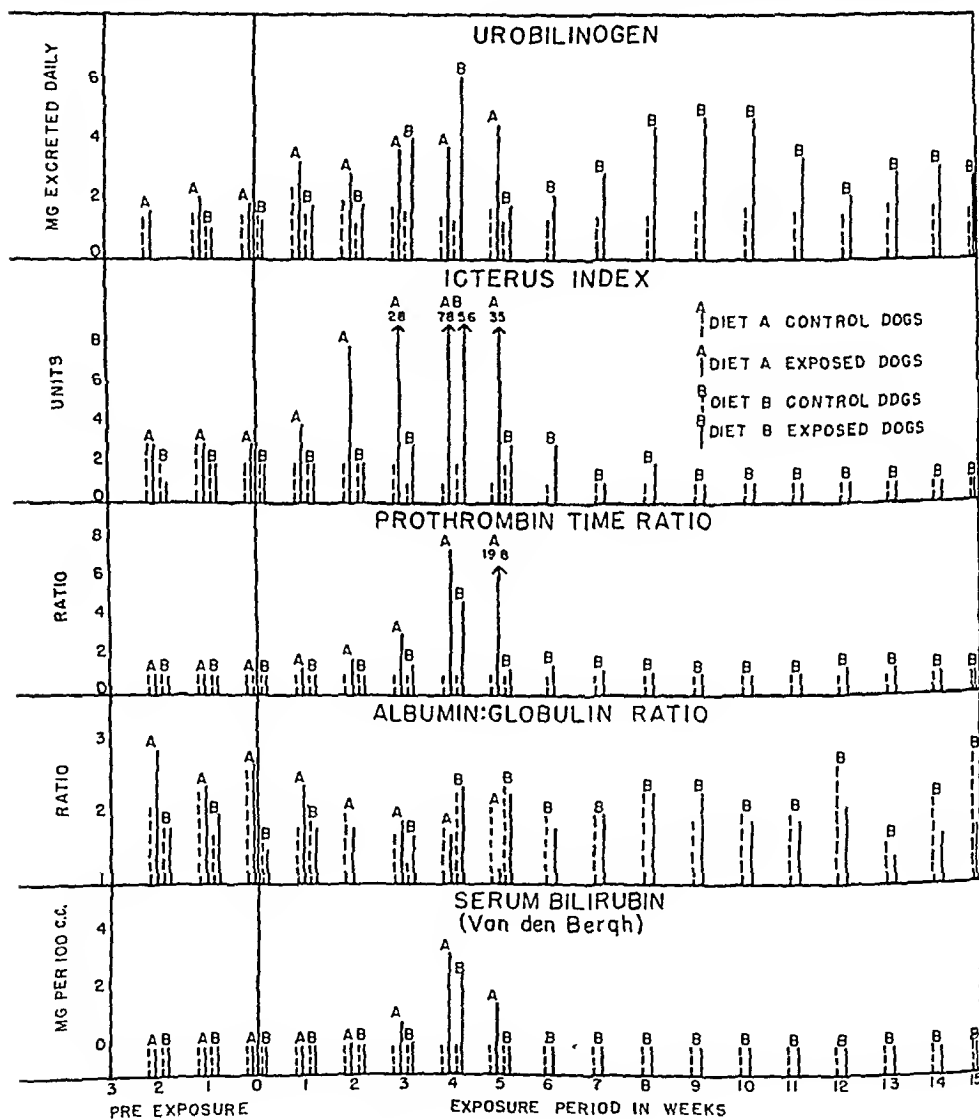


Fig. 2.—Liver function tests.

with hematoxylin-azure cosinate,¹⁷ the modified van Gieson stain for hemoglobin,¹⁸ and the acidulated ferrocyanide reaction for iron. Frozen sections were stained for fat according to the technique of Lillie and Ashburn.¹⁹ Other special stains such as Mallory's aniline blue collagen stain²⁰ and Laidlaw's silver stain for reticulum²⁰ were used as desired.

TABLE I. RESULTS OF LIVER FUNCTION TESTS ON DOGS ON GROUP I (DIET A)

WEEKS ON TEST	BROMOPHALEIN RETENTION (PER CENT)	ROSE BENGAL RETENTION (PER CENT)	BILIRUBIN RETENTION (PER CENT)	SERUM PHOSPHATASE (UNITS PER 100 G.G.)	PROTHROMBIN TIME: RATIO	URINARY UROBILINOGEN (MG. PER DAY)	COMMENTS
<i>Controls (Average of Dogs 12, 13, and 16)</i>							
Pre exposure	6.2	11.2	11.2	1.1	1.0	1.5	
1	1.9	8.7	8.9	2.3	1.0	2.4	
2	3.2	8.0	7.9	2.3	1.0	2.0	
3	2.6	7.6	10.7	2.6	1.0	1.8	
4	13.2	16.2	14.1	3.1	1.0	1.6	
5	1.6	12.9	8.6	2.8	1.0	1.7	
6	2.5	19.5	15.4	3.8	1.0	1.8	
7	-	8.6	-	3.2	1.0	2.1	Normal livers
<i>Dog 11</i>							
Pre exposure	5.7	12.3	15.5	1.4	1.1	1.9	
1	13.2	21.0	9.8	1.1	1.4	3.5	
2	62.0	41.5	20.0	3.8	2.2	3.6	
3	65.0	56.8	6.8	4.7	4.4	3.9	
4	-	-	-	-	8.2	4.8	Jaundice noted Precirrhotic liver
<i>Dog 11</i>							
Pre exposure	4.1	8.3	16.5	1.9	1.0	1.4	
1	13.6	19.5	22.7	6.3	1.2	3.7	
2	53.1	41.6	16.0	7.3	1.7	3.6	
3	66.7	59.5	81.0	6.1	3.2	4.8	
4	-	-	-	16.6	10.7	2.1	Jaundice noted Precirrhotic liver
<i>Dog 15</i>							
Pre exposure	4.8	1.7	13.1	1.5	1.0	2.0	
1	11.1	13.5	13.6	2.7	1.1	3.7	
2	-	-	-	5.2	1.6	2.4	Jaundiced at death Fatty metamorphosis of liver
<i>Dog 19</i>							
Pre exposure	0.0	10.1	4.0	1.5	1.0	2.5	
1	9.1	13.3	0.0	5.1	1.4	2.6	
2	39.0	31.8	14.6	5.1	1.8	2.2	
3	50.0	30.2	28.6	3.5	1.9	2.5	
4	56.0	73.0	11.5	4.7	3.6	4.3	
5	-	16.2	-	12.7	15.3	1.6	Jaundice noted Precirrhotic liver

TABLE II. RESULTS OF LIVER FUNCTION TESTS ON DOGS OF GROUP II (DIET B)

WEEKS ON TEST	BROMSULFALEIN RETENTION (PER CENT)	ROSE BENGAL RETENTION (PER CENT)	BILIRUBIN RETENTION (PER CENT)	SERUM PHOSPHATASE (UNITS PER 100 G.G.)	PROTHROMBIN TIME RATIO	URINARY UROBILINOGEN (MG. PER DAY)	COMMENTS
<i>Controls (Average of Dogs 21, 24, and 25)</i>							
Pre-exposure	4.0	11.3	12.7	2.3	1.0	1.5	
1	2.4	0.0	14.5	2.9	1.0	1.5	
2	2.5	0.9	10.3	2.7	1.0	1.2	
3	7.5	1.3	11.6	3.0	1.0	1.7	
4	5.7	-	-	2.6	1.0	1.3	
5	4.8	3.2	6.7	2.6	1.0	1.3	
6	9.2	6.2	9.1	3.4	1.0	1.3	
7	4.2	9.5	11.3	2.0	1.0	1.4	
8	3.7	11.3	7.1	2.9	1.0	1.5	
9	6.4	3.4	8.3	2.8	1.0	1.6	
10	5.6	5.4	10.5	3.1	1.0	1.8	
11	3.9	7.3	8.1	4.5	1.0	1.6	
12	3.6	5.7	6.3	5.0	1.0	1.6	
13	5.5	3.5	6.0	6.1	1.0	1.9	
14	4.0	9.9	5.6	5.3	1.0	1.8	
15	3.4	6.4	-	5.7	1.0	1.7	Normal livers
<i>Dog 20</i>							
Pre-exposure	2.4	6.9	10.6	1.7	1.0	1.4	
1	3.3	0.0	16.7	3.5	1.1	2.0	
2	8.4	27.3	9.1	2.8	1.0	1.8	
3	64.4	34.7	24.3	4.1	1.9	3.8	
4	89.0	-	-	8.2	9.4	10.4	Jaundice noted Precirrhotic liver

TABLE II. RESULTS OF LIVER FUNCTION TESTS ON DOGS OF GROUP II (DIET B)

WEEKS ON TEST	BROMSULFALEIN RETENTION (PER CENT)	ROSE BENGAL RETENTION (PER CENT)	BILIRUBIN RETENTION (PER CENT)	SERUM PHOSPHATASE (UNITS PER 100 C.C.)	PROTHROMBIN TIME RATIO	URINARY UROBILINOGEN (MG. PER DAY)	COMMENTS
Pre-exposure	4.0	11.3	12.7	2.3	1.0	1.5	
1	2.4	0.0	14.5	2.9	1.0	1.5	
2	2.5	0.9	10.3	2.7	1.0	1.2	
3	7.5	1.3	11.6	3.0	1.0	1.7	
4	5.7	-	-	2.6	1.0	1.3	
5	4.8	3.2	6.7	2.6	1.0	1.3	
6	9.2	6.2	9.1	3.4	1.0	1.3	
7	4.2	9.5	11.3	2.0	1.0	1.3	
8	3.7	11.3	7.1	2.9	1.0	1.4	
9	6.4	3.4	8.3	2.8	1.0	1.5	
10	5.6	5.4	10.5	3.1	1.0	1.6	
11	3.9	7.3	8.1	4.5	1.0	1.8	
12	3.6	5.7	6.3	5.0	1.0	1.6	
13	5.5	3.5	6.0	6.1	1.0	1.6	
14	4.0	9.9	5.6	5.3	1.0	1.9	
15	3.4	6.4	-	5.7	1.0	1.8	
Pre-exposure	2.4	6.9	10.6	Dog 20	1.0	1.7	Normal livers
1	3.3	0.0	16.7	1.7	1.0	1.4	
2	8.4	27.3	9.1	3.5	1.1	2.0	
3	64.4	34.7	24.3	2.8	1.0	1.8	
4	89.0	-	-	4.1	1.9	3.8	
				8.2	9.4	10.4	Jaundice noted Precirrhotic liver

case of Dog 22, on Diet B, the moderate elevation of serum phosphatase, for the first eight weeks, rose to a markedly high level during the rest of posture period. In this latter period, the other tests either returned to normal or had decreased values.

Prothrombin Time Ratio. The rise in the prothrombin time ratio (values of 2 being considered abnormal) generally paralleled the increasing retention of bromsulfalein, except in the case of Dog 22. However, without superevidence, the increases noted in the ratio during the first three weeks were sufficient to be conclusive. A marked increase in this ratio was usually prior to the death of the animal, when liver damage was indicated by other tests. This test is technically more difficult than the bromsulfalein test. Wide variations in clotting time may occur unless the amount of calcium added to the blood is carefully controlled.

Urobilinogen. This test did not appear to be as sensitive or as reliable as the bromsulfalein retention test. Values exceeding the excretion of 10 mg of urobilinogen were considered abnormal. Considerable variation existed among the dogs regarding the time required for excreting high amounts of urobilinogen. The data obtained indicated that spot analysis of the urine was satisfactory for urobilinogen estimations. Excretion of urobilinogen for most dogs reached high levels for one or two days and then returned to normal. Useful information with this test can only be obtained by daily analysis of four-hour samples of urine.

Van Bergh Reaction.—This test was of little value in detecting early liver damage, the direct and indirect reaction being obtained only when the dogs were jaundiced and near death. Bilirubin is not found in the blood of normal dogs, so appreciable amounts in the blood can only be expected in cases of severe liver damage.

Serum Icterus Index.—In Group I, the icterus index rose above the normal values during the exposure period but did not reach high values until death was near. Results in Group II were less conclusive, and the test was considered inferior to the others used.

Fibrinogen and Albumin:Globulin Ratio.—Neither of these tests showed changes which proved to be statistically significant. However, there was usually a decrease in fibrinogen prior to death of the animal.

PATHOLOGIC EXAMINATION

Dog 15, dying after ten days, showed marked fatty metamorphosis in the central two-thirds of the hepatic lobules.

The livers of Dogs 11, 14, 19, 20, and 23, dying within four to seven weeks, were precirrhotic. This was characterized by patchy and periportal proliferation of small basophilic spindle cells forming poorly margined bands extending between adjacent portal areas and to the capsule. These cells formed strands or small tubules which at times closely resembled bile ducts. In addition, these bands had a few collagen and silver-positive reticulum fibers and were infiltrated by cells, chiefly lymphocytes.

RESULTS AND DISCUSSION

The data obtained from the tests are shown in Figs. 1 and 2 in which each value represents the average for all dogs surviving at that time.

The results of the liver function tests which gave early evidence of hepatic damage are shown in Tables I and II. Since the tests were performed on definite days of the week, some of the values are missing during the last week of exposure, due to the death or sacrifice of the animal.

Bromsulfalein Retention Test.—This test was the most sensitive and consistent of those studied. With the procedure followed in this experiment, it was concluded that, generally, retentions up to 10 per cent may be expected in normal dogs. Retentions from 10 to 15 per cent are suggestive of liver damage, and those above 15 per cent are definitely abnormal. All of the exposed dogs fed Diet A showed high retention of the dye by the end of the second week, three of the animals (Dogs 11, 14, and 15) having been in the suggestive range the previous week. Abnormal retention in Dogs 20 and 23, fed Diet B, occurred in the third week and in Dog 22 during the fourth week of exposure. The marked retention of the dye was noted soon after the dogs could not retain the food. For Dog 22, which retained its food, the abnormally high retention values reached the peak at the end of the sixth week and never exceeded the high values found in the rest of the dogs.

Rose Bengal Retention Test.—This test closely paralleled the bromsulfalein test. However, the values obtained on the control dogs varied more widely due to the difficulties in measuring dye concentrations in the presence of varying degrees of turbidity. It was found necessary to take initial blood samples for each dog prior to injection of the dye, the plasma being used as the blank in the spectrophotometer. Retentions up to 15 per cent were considered normal; 15 to 20 per cent, suggestive; and above 20 per cent, definitely indicative of liver damage. Ivy and Roth²¹ have likewise reported that the rose bengal test in the dog gave comparable results to those obtained by the bromsulfalein test.

Bilirubin Retention Test.—This test was less sensitive and more erratic than either of the dye tests. Generally, abnormal retentions (those above 15 per cent) were not observed until one week after the dye tests had indicated liver injury. Due to the rapid excretion of bilirubin by dogs, the per cent retention seldom reached high levels even in cases of severely damaged livers. Drill and Ivy⁷ concluded that this test was of little value in detecting liver damage in dogs poisoned by carbon tetrachloride. Smith and associates⁶ have reported, however, an abnormal retention of bilirubin and of rose bengal in most of their chronically selenium-poisoned cats and rabbits.

Serum Phosphatase.—This test was found to be useful in detecting liver injury. A moderate rise in phosphatase activity (values above 4 units per 100 c.c. of serum being considered abnormal) was generally noted for all the dogs during the first week of exposure. High elevations were not noted until the fourth week of exposure. In the absence of normal values for individual subjects, this test would not appear to be as conclusive in making an early diagnosis of liver damage as either the bromsulfalein or rose bengal retention tests.

In the case of Dog 22, on Diet B, the moderate elevation of serum phosphatase, noted for the first eight weeks, rose to a markedly high level during the rest of the exposure period. In this latter period, the other tests either returned to the normal or had decreased values.

Prothrombin Time Ratio.—The rise in the prothrombin time ratio (values above 1.2 being considered abnormal) generally paralleled the increasing retention of bromsulfalein, except in the case of Dog 22. However, without supporting evidence, the increases noted in the ratio during the first three weeks were not sufficient to be conclusive. A marked increase in this ratio was usually noted prior to the death of the animal, when liver damage was indicated by all the other tests. This test is technically more difficult than the bromsulfalein retention test. Wide variations in clotting time may occur unless the amount of oxalate added to the blood is carefully controlled.

Urinary Urobilinogen.—This test did not appear to be as sensitive or as reliable as the bromsulfalein retention test. Values exceeding the excretion of 3 mg. per day of urobilinogen were considered abnormal. Considerable variation existed among the dogs regarding the time required for excreting high amounts of urobilinogen. The data obtained indicated that spot analysis of the urine is not satisfactory for urobilinogen estimations. Excretion of urobilinogen for individual dogs reached high levels for one or two days and then returned to normal. Useful information with this test can only be obtained by daily analysis of twenty-four hour samples of urine.

Van den Bergh Reaction.—This test was of little value in detecting early signs of liver damage, the direct and indirect reaction being obtained only when the dogs were jaundiced and near death. Bilirubin is not found in the blood of normal dogs, so appreciable amounts in the blood can only be expected in cases of severe liver damage.

Icterus Index.—In Group I, the icterus index rose above the normal values early in the exposure period but did not reach high values until death was near. Results in Group II were less conclusive, and the test was considered inferior to some of the others used.

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PATHOLOGIC EXAMINATION

Dog 15, dying after ten days, showed marked fatty metamorphosis in the central two-thirds of the hepatic lobules.

The livers of Dogs 11, 14, 19, 20, and 23, dying within four to seven weeks, were precirrhotic. This was characterized by patchy and periportal proliferation of small basophilic spindle cells forming poorly margined bands extending between adjacent portal areas and to the capsule. These cells formed strands or small tubules which at times closely resembled bile ducts. In addition, these bands had a few collagen and silver-positive reticulum fibers and were infiltrated by cells, chiefly lymphocytes.

Scattered through the parenchyma, often near the interportal bands, there were groups of basophilic regenerating liver cells. An occasional cell in these groups showed partial cytoplasmic oxyphilia. More often there were plugs of inspissated bile in the canaliculi.

Elsewhere, the fat-laden liver cells were usually swollen, oxyphilic, and occasionally frankly necrotic. Hemosiderin, generally in small amounts, was present in scattered Kupffer cells and less often in hepatic cells.

The liver of Dog 22, killed after fifteen weeks, was grossly nodular and cirrhotic. This was characterized, microscopically, by coarse fibrous trabeculations, irregularly traversing the liver, distorting the lobular architecture, and segregating nodules of basophilic liver cells. These bands contained small bile ducts and were infiltrated by many cells, chiefly lymphocytes and lipochrome-laden macrophages. Occasionally, small groups of liver cells, some of which were necrotic, were included in these bands.

The livers of the control animals showed no significant changes.

The other changes to be noted in the dogs dying within four to seven weeks were the presence of a few bile casts in the renal tubules and hemosiderin in scattered littoral cells, especially in the splenic pulp. These changes were absent in the dog killed at fifteen weeks.

SUMMARY AND CONCLUSIONS

Xylidine at a moderately low concentration is an effective agent in producing jaundice and fatty livers in dogs. With prolonged exposure, the livers pass through a precirrhotic to a cirrhotic stage. Microscopic examination of the liver showed evidence of parenchymal damage with no evident impairment in the cells of the reticulo-endothelial system, since these retain their function of phagocytosis of iron-positive pigment.

The bromsulfalein retention test was found to be the most reliable one in detecting liver damage. The rose bengal retention test was almost as sensitive but had certain disadvantages which made it less desirable.

The serum phosphatase activity and prothrombin time ratio were of equal value but less conclusive than the dye retention tests.

The bilirubin retention and urinary urobilinogen excretion tests gave indications of liver injury but were less consistent than the dye retention, serum phosphatase, or prothrombin time ratio tests.

Icterus index determinations may be of some value whereas the van den Bergh, fibrinogen, and albumin:globulin ratio estimations do not appear to yield information early enough to aid in the diagnosis of liver injury.

This order of efficiency agrees very well with the results obtained by Neefe and associates⁵ in their work on liver damage produced in human subjects by administration of yellow fever vaccine and with the findings of Drill and Ivy⁷ in carbon tetrachloride poisoned dogs.

Acknowledgment is made to Mrs. Virginia B. Hauff, for her assistance throughout this experiment.

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STUDY OF ABSORPTION FROM CRYSTALLINE INSULIN PELLETS AND SOLUTIONS AT VARIOUS SITES IN RABBITS

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THE study herein reported was initiated primarily to determine if absorption from insulin pellets varied at different sites of implantation. Insulin pellet implants have been reported;^{1,2} however, to our knowledge there is no information relative to insulin implants in the spleen. It was believed that study of splenic implants would be of interest inasmuch as the venous drainage from both the spleen and pancreas goes mainly to the liver via the portal system. Therefore, absorption from splenic implants would closely follow the route of normal endogenous insulin after its release by the pancreas. Any hepatic regulatory effect on insulin would be expected to play its part in absorption from splenic implants, whereas little or no effect from the liver would be expected in the case of intramuscular or subcutaneous pellets.

MATERIAL AND METHODS

Crystalline zinc insulin with a potency of 25.89 units per milligram was used throughout the experiments. Pellets were made by compressing the material as tightly as possible in a small mechanical hand press. The pellets had a diameter of approximately 2 mm. and a thickness of $\frac{1}{2}$ to 1 mm. depending on the weight of insulin used. The rabbits used in the pellet implantation experiments were New Zealand Whites weighing from 3.5 to 4.5 kilograms. They were starved for a twenty-four hour period preceding the experiment and during the blood sampling period, but water was given.

Sodium pentothal was used as the anesthetic. Each rabbit was given an initial intravenous injection of 2 c.c. of 2 per cent solution fairly rapidly. This resulted in light anesthesia. Additional amounts of sodium pentothal solution were then given slowly until surgical anesthesia was reached. The rabbits tolerated pentothal very well. Only one rabbit out of forty-one was lost from respiratory failure and that was the third animal used in the series. The amount of 2 per cent sodium pentothal solution used varied from 2.5 to 10 c.c. with an average of 4.9 c.c. per rabbit.

The splenic implants were performed as follows. The upper abdomen was opened with a midline incision, and the spleen was brought to the surface. The arterial supply was clamped off lightly and a small incision made through the fibrous capsule. The pellet was then inserted deeply in the splenic pulp and the capsule closed with a single silk suture. The clamp was then removed, and the abdomen was closed with linen sutures. The skin was closed by metal clips. A bandage was wrapped around the abdomen to prevent trauma to the wound from the animal's claws.

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The intramuscular pellets were inserted in the substance of the trapezius muscle, and the muscle incision was closed with a silk suture. The skin incision was closed with metal clips. The subcutaneous pellets were implanted in the scapular area, and the skin incision was closed with metal clips. Controls were run with 5 c.c. of 2 per cent sodium pentothal solution to rule out any effect of the anesthetic on the blood sugar level. A second series of controls were run using starch pellets implanted in the spleen to rule out any effect of the operative procedure on the blood sugar. Blood samples were taken from the marginal vein of the ear just before the operation and at four, five, twenty-four, and thirty hours after the implant was made. Glucose determinations were made by the micro method of Hagedorn-Jensen.

A solution containing 50 units per cubic centimeter of the crystalline insulin was made up in slightly acidulated water containing 0.1 per cent phenol. A group of ten fasting rabbits were injected subcutaneously with 0.8 units each and a second group of ten with the same amount intramuscularly. Blood samples were taken before injection and at three-fourths, one and one-half, two and one-half, and four hours following injection. The groups were reversed a week later.

RESULTS AND DISCUSSION

The results of the pellet implants and anesthetic controls are shown in Table I and in Fig. 1.

The sodium pentothal solution given intravenously had very little effect on the blood sugar level. Blood sugar values at four and five hours were slightly but not significantly lower than before the anesthetic was given.

TABLE I

EXPERIMENTAL PROCEDURE	TOTAL NUMBER OF ANI- MALS IN EXPERI- MENT	DEATHS WITHIN 24 HOURS		DEATHS FROM 24 TO 48 HOURS		KILLED INSULIN SHOCK FROM 24 TO 48 HOURS		SURVIVAL AFTER 48 HOURS		PELLET SIZE (MILLIGRAMS)	
		NO. (%)		NO. (%)		NO. (%)		NO. (%)		VARIATION AVERAGE	
		NO.	(%)	NO.	(%)	NO.	(%)	NO.	(%)		
Insulin pellets											
Subcutaneous	10	8	80	0		1	10	1	10	2.9 to 2.1	2.56
Intramuscular	10	4	40	0		2	20	4	40	5.3 to 2.1	3.07
Intrasplenic	10	4	40	1	10	1	10	4	40	6.5 to 3.0	3.65
Starch pellets											
Intrasplenic	5	0		0				5	100	10.0 to 5.0	8.00
Pentothal											
Intravenous	5	0		0				5	100		
5 c.c. 2 per cent											

The operation with starch implants intrasplenicly resulted in a moderate increase in the blood sugar in 80 per cent of the animals four and five hours after the operation. All of the controls survived during the thirty-hour observation period. The subcutaneous insulin pellet implants produced a pronounced drop in the blood sugar to an average value of 49.9 mg. per cent glucose at four hours and 46.4 mg. per cent glucose at five hours after the operation. Eight out of ten rabbits died in insulin shock within the twenty-

four hour period following the implantation. Examination of the pellets after the death of the animals revealed that they had become very soft and, in several cases, had lost most of their shape.

The intramuscular insulin implants resulted in a decrease in blood sugar level to an average value of 60.6 mg. per cent glucose at four hours and 57.4 mg. per cent glucose at five hours after the implantation. Four out of ten rabbits died in insulin shock within twenty-four hours of the operation. Examination of the pellets, after the death of the rabbits, revealed that they retained their shape fairly well but were soft. The twenty-four and thirty-hour blood sugar levels on the six surviving animals were 23.4 and 11 mg. per cent lower than the initial value showing a definite hypoglycemic action for at least thirty hours.

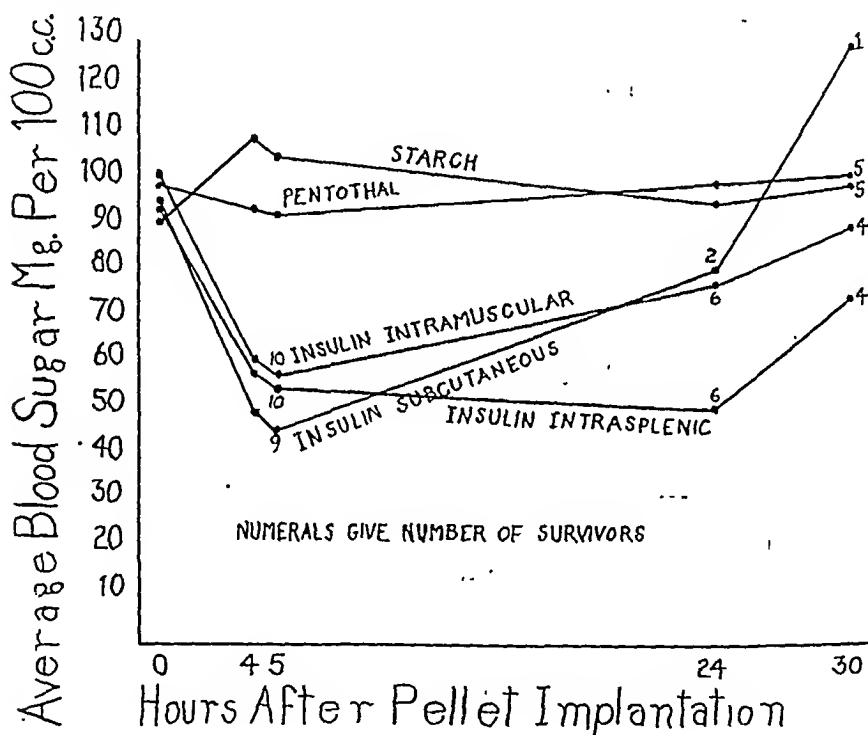


Fig. 1.

The intrasplenic insulin implants gave a hypoglycemic effect very similar to the intramuscular implants at the four- and five-hour periods, the blood sugars being 58.8 and 55.3 mg. per cent glucose, respectively. The twenty-four hour value (six rabbits) was 51 mg. per cent glucose, while the thirty-hour value (four rabbits) was almost 20 mg. per cent lower than the initial level. The splenic implants hence gave a somewhat longer insulin release than the intramuscular pellets. There was very little change in the shape of the pellets in the spleen although they became quite soft. There was no evidence of any regulatory action by the liver on the insulin released from splenic

TABLE II

EXPERIMENTAL PROTOCOL	BLOOD SUGAR (MILLIGRAMS PER CENT GLUCOSE)				
	INITIAL	2 1/2 HOURS	11 1/2 HOURS	21 1/2 HOURS	4 HOURS
0.8 Units insulin subcutaneous	106.1	78.4	70.2	74.3	101.5
0.8 Units insulin intramuscular	103.7	76.3	72.3	84.9	109.9

implants. The results of the intramuscular and subcutaneous injections are shown in Table II. The two and one-half hour value with the intramuscular injection is somewhat higher than with the subcutaneous injection. There are, however, no differences between the two curves either in the rapidity of onset or depth of the hypoglycemia. This result would indicate that the more rapid absorption from subcutaneous insulin pellets is probably due to mechanical factors with a greater tendency for the subcutaneous pellet to be disrupted. This is in agreement with the appearance of the pellets after the deaths of the rabbits.

CONCLUSIONS

Subcutaneous insulin pellet implants in rabbits result in a more rapid and intense hypoglycemic action than intramuscular or intrasplenic implants. This is probably due to mechanical factors that cause a more rapid breakdown of the pellet.

Intrasplenic insulin pellet implants produce a more prolonged insulin action than intramuscular implants. There is apparently no appreciable hepatic regulation on the insulin released from the splenic implants.

There are no marked differences in the insulin action in rabbits between intramuscular and subcutaneous injections with regard to speed of onset and depth of hypoglycemia.

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THE NATURE OF THE ACTION OF DIMETHYLAMINOETHYL
BENZHYDRYL ETHER HYDROCHLORIDE (BENADRYL):
EFFECTS UPON THE HUMAN EYE

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THE antihistamine action of Benadryl has been well established,¹⁻¹³ and upon this much of its weaker antispasmodic effect may secondarily depend.^{3, 8, 20, 21} Because of its antagonism to the depressor and spasmogenic influence of acetylcholine, an atropine-like activity has been claimed.^{1, 3, 10, 16, 22} The dryness of the mouth sometimes seen clinically may be a manifestation of this character. On the other hand, others have observed an increase in the pressor action of epinephrine,²² although this has not been confirmed at the bedside.¹⁶ Symptoms and signs of sympathomimetic action are conspicuously absent in patients who have taken the drug in quantities up to 600 mg. daily.¹⁶ No increase in pulse rate or blood pressure is evident; dryness of the mouth is rare; and palpitation has not been noted. "Jitteriness" and "inward nervousness" are not only absent, but placidity and even drowsiness may occur;¹⁶ when the drug is given orally or intravenously, it causes no dilatation of the pupil of the eye.¹⁰ Effort has been made in the present study to extend the observations in connection with the oral administration of the drug and to record the effects of the alkamine ether following its topical application to the eye, thereby clarifying certain points regarding the fundamental nature of its action.

METHODS AND RESULTS

The Effect of Orally Administered Benadryl.—The pupils of twenty ambulatory subjects, without known disease of the autonomic nervous system or organic disease of the eye, were observed before, periodically during, and after the use of benadryl orally in daily doses of 150 to 400 mg. for periods of time ranging from three to twelve weeks. Throughout the period of study, a constant diet was used, other drugs were not employed, and normal freedom of the hospital wards was allowed. Measurements of pupillary diameters with an especially adapted millimeter ruler were carried out under constant conditions of lighting, while the attention of the patient was directed to an object, the character and position of which did not vary.

No perceptible change in pupillary size nor in the ability of the subject to read a Snellen chart were observed in any one of these subjects as a result of the administration of benadryl.

From the New York Medical College, Metropolitan Hospital Research Unit.

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The Effect of Topically Applied Benadryl.—For these experiments, sixty ambulatory subjects were chosen at random, save for the exclusion of anyone with an organic disease of the eye or a clinically recognizable disturbance of the autonomic nervous system. The ages of these individuals ranged from 21 to 65 years. All applications were made to the right eye; the "fore" reading and readings made on the left eye all served as controls. At no time in any one experiment was the size or accommodative capacity of the left eye perceptibly affected. One drop of a fresh 0.5 per cent aqueous solution was instilled at ten-second intervals for three doses, unless otherwise noted. All readings were taken at 0-, 15-, and 60-minute intervals, respectively, in relation to the instillation of the drug.

The Influence of Benadryl Alone: A definite increase in the size of the pupil was observed in forty-eight of the sixty subjects, averaging approximately 1.9 mm. at the end of one hour (Table I). In the remaining twelve, no change was observed; the washing out effect of the excessive flow of tears may have played a part in this failure to react.

As a rule, a maximum dilatation was present by the end of an hour and had begun to decrease quite noticeably within two hours. In only four patients was any dilatation still measurable at the end of twenty-four hours.

TABLE I. CHANGES IN VISUAL APPARATUS OF SIXTY SUBJECTS FOLLOWING TOPICAL APPLICATION OF BENADRYL (0.5 PER CENT AQUEOUS SOLUTION)

	INCREASED IN	DECREASED IN	UNCHANGED IN
Papillary size	48	0	12
Visual acuity	0	12	48
Accommodation	0	43	17

Visual acuity, as determined by the Snellen chart read at a 20-foot distance, was decreased in twelve subjects and unaltered in forty-eight (Table I). No attempt was made to correlate these findings with the presence or absence of astigmatism. Capacity for accommodation was tested separately for each eye before and sixty minutes after the instillation of benadryl with the aid of a Prinz accommodation ruler and a Jaeger reader No. 3. In each instance, the chart was moved toward the subject at a uniform speed from an easy reading distance. The point at which the chart became blurred and was no longer readable was accepted as the "limit of accommodation." Both before and after benadryl, the results of from three to six readings were averaged to obtain the final figure recorded. Patients unable to give reliable results prior to the instillation of benadryl were eliminated from the experiment. Forty-three of the sixty patients showed a decreased capacity for accommodation. The average "limits of accommodation" for these forty-three subjects before and after benadryl were 10.3 and 13.1 cm., respectively. Corresponding figures determined for the entire group of subjects were 10.7 and 12.7 cm., respectively.

The Effect of Epinephrine Upon the Action of Benadryl: In these and subsequent experiments, three drops of benadryl were used in the right eye, whereas only one drop of each of the other solutions, respectively, was em-

ployed. Whenever a second agent was tested (unless otherwise stated), it was given first, and readings were made at 0, 15, and 60 minutes, respectively. Immediately after the sixty-minute period, the benadryl solution was added with additional observations at 75 and 120 minutes, respectively.

The results of these and all subsequent experiments on the size of the pupil, for which fifteen subjects were used, are summarized in Table II. In all instances, the size of the pupils in the "fore" period served as a control, and the diameter found has been expressed arbitrarily as 100. The "after" readings have been recorded as a percentage figure of this "normal." In six patients benadryl and epinephrine (0.001 per cent) were instilled, one immediately after the other, to a total of three drops for each. The total ensuing dilatation was no greater than with benadryl alone but reached its maximum earlier, that is, in fifteen minutes rather than in one hour.

The mydriasis following a single drop of epinephrine was slight in degree and did not influence the response to subsequently instilled benadryl (Table II).

The Effect of Homatropine Upon the Action of Benadryl: One drop of a 1.0 per cent aqueous solution of homatropine sulfate caused an average increase of 156 per cent in the size of the pupil of the right eye of each of fifteen subjects. The subsequent administration of benadryl caused a small additional average widening (169 per cent) (Table II), although two of the fifteen patients did not respond at all.

TABLE II. COMPARISON OF EFFECTS UPON PUPILLARY SIZE OF BENADRYL AND OTHER AGENTS, SINGLY AND IN COMBINATION

SUB- JECT NO.	BENADRYL (0.5%)	EPINEPH- RINE HCl (0.001%)	EPINEPH- RINE HCl PLUS BENADRYL	HOM- ATROPINE SO ₄ (1.0%)	HOM- ATROPINE SO ₄ PLUS BENADRYL	ESERINE SO ₄ (0.5%)	ESERINE SO ₄ PLUS BENADRYL
1	100	100	100	200	200	25	22
2	108	108	100	133	140	33	33
3	160	102	140	175	200	17	40
4	150	100	133	150	175	40	48
5	100	100	100	150	150	38	38
6	138	110	140	120	150	67	67
7	127	100	129	167	187	25	55
8	150	113	125	175	195	40	63
9	120	100	140	160	165	33	42
10	125	100	133	140	147	50	55
11	120	100	133	167	177	33	33
12	140	108	138	160	166	33	50
13	124	104	122	180	192	57	67
14	120	105	120	145	163	28	44
15	123	102	119	128	130	51	56
Average	127.0	104.1	124.8	156.6	169.1	38.0	47.6

The Effect of Eserine Upon the Action of Benadryl: Eserine sulfate, preferably termed physostigmine sulfate (0.5 per cent) produced a characteristic miosis in which the average diameter of the pupillary aperture was reduced to 38.0 per cent of its former size. In five of fifteen patients, the subsequent application of benadryl, under the conditions of the experiment, had no influence upon the pupillary opening, but the average increase in diameter for the entire group was of sufficient magnitude to augment the size of the pupil from 38.0 to 47.6 per cent of its initial value (Table II).

CONCLUSION

While certain variations of the previously mentioned procedures might permit more accurate measurements of the effects of benadryl upon the eye, the results of the dosage, time of application, frequency of observation, and methods of measurement arbitrarily employed have afforded data capable of clear interpretation. The partial paralysis of accommodation, the slight inhibition of the miosis caused by eserine, and the furthering effect of benadryl upon the mydriasis produced by epinephrine and atropine all seem to point toward an atropine-like or vagal-paralyzant effect of benadryl when topically applied to the eye.

SUMMARY

1. Orally administered dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) in doses up to 400 mg. daily does not influence the size of the pupil of the eye.

2. Benadryl topically applied to the bulbar conjunctiva in 0.5 per cent aqueous solution causes within fifteen minutes a moderate mydriasis of readily measurable proportions; this becomes maximal within one hour. The capacity for accommodation is simultaneously decreased.

3. Epinephrine accelerates the action of benadryl upon the pupil but under the conditions of these experiments does not increase its intensity.

4. Benadryl furthers the mydriatic effect of homatropine and lessens the miosis produced by eserine.

5. The probable nature of the ocular action of benadryl is mentioned.

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LABORATORY METHODS

CONSTANT FLOW GASSING CHAMBERS: PRINCIPLES INFLUENCING DESIGN AND OPERATION

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INTRODUCTION

ALTHOUGH dynamic (or constant flow) chambers for the experimental gassing of animals or human subjects have been described previously,¹⁻¹⁰ the basic principles underlying the operational behavior of such chambers have not been discussed. It is the purpose of this paper to present such a discussion. Experimental data for various gases are given in verification of the principles described.

DESCRIPTIVE

A dynamic chamber is one through which air is continuously drawn at a fixed rate. The agent to be investigated is introduced into the affluent air as a vapor, smoke, dust, or fog. Animals to be exposed are introduced into the chamber through a door, after the desired concentration has been established. The constant stream of air through the chamber renews the oxygen supply for the animals and removes the exhaled moisture and carbon dioxide. Concentrations may be maintained by this system with comparative ease.

However, it is essential that the agent be dispersed at a constant rate through the period of operation. The upper limit of concentration obtainable is fixed by the physical characteristics of the disseminated chemical and the method of dispersion. A discussion of methods or apparatus for the dispersion of agents in various states is not within the scope of the present report.

The flow of air through the chamber is maintained by a pump, usually in the effluent line, and is measured by an accurately calibrated orifice meter, rotameter, or similar device. To insure an even air flow, buffer tanks may be installed to eliminate the effect of pump pulsations. Since with the pump in the effluent line the pressure within the chamber is always slightly less than atmospheric, leakages outward from the chamber cannot occur. Thus, extremely toxic compounds can be biologically assayed without danger to the experimenter.

ESTIMATION OF CONCENTRATION

Concentrations of agents in the chamber may be either calculated theoretically or determined by analysis. The theoretical or "nominal" concentration, as it has been called in Chemical Warfare Service laboratories for many years, is calculated from the quantity of agent introduced into the chamber per minute and the rate of air flow through the chamber, as follows:

$$\text{Nominal concentration in milligrams per liter} = \frac{\text{agent flow in milligrams per minute}}{\text{air flow in liters per minute}}$$

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The validity of such nominal concentrations depends on the accuracy of measurement of both the rate of flow of the agent into the chamber and the rate of flow of air through the chamber, *always provided that both of these flows are constant.*

The analytical concentration is measured by chemical or physical analysis of a sample of air drawn from the chamber. If the collection of the sample is complete and the method of analysis accurate, the analytical concentration is the actual concentration within the chamber at the time of sampling. Lack of agreement between the analytical and nominal concentrations may be due to one or more of the factors discussed herein.

EQUILIBRATION

When a vapor or gas is introduced at a uniform rate into a chamber through which there is a continuous flow of air, the concentration within the chamber increases until it is practically constant. If it is assumed that perfect mixing with the chamber air occurs, the concentration at any time may be calculated by the following modification of the usual ventilation equation¹²:

$$C = \frac{w}{b} (1 - e^{-\frac{bt}{a}}) \quad (1)$$

Where C = the concentration in milligrams per liter at time t

w = milligrams of agent introduced per minute

a = the volume of the chamber in liters

b = the volume of air passing through the chamber each minute.

The percentage of the desired concentration $\frac{w}{b}$ obtained in time t is then given by:

$$\text{Per cent} = 100 (1 - e^{-\frac{bt}{a}}) \quad (2)$$

From Equation 1, it is apparent that the chamber concentration, at first, rises rapidly and then approaches a constant value at infinite time. Consequently, for practical purposes, animals may be introduced into the chamber when the concentration reaches 99 per cent of the theoretically constant value, since the rise in concentration from that point is less than 1 per cent for any finite exposure period. The time required for equilibration of the chamber to 99 per cent may be calculated by setting Equation 2 equal to 99:

$$\begin{aligned} 100 (1 - e^{-\frac{bt}{a}}) &= 99 \\ \text{or} \quad e^{-\frac{bt}{a}} &= \frac{100 - 99}{100} = 0.01 \end{aligned}$$

which, when transformed into logarithmic form, becomes:

$$-\frac{bt}{a} = \ln 0.01 = -4.6052$$

$$\text{and} \quad t_{99} = 4.6052 \frac{a}{b} \quad (3)$$

Thus, if the size of the chamber (a) and the rate of air flow (b) are known, the time for reaching 99 per cent of the theoretical concentration at infinite time can be estimated easily from Equation 3.

This equation may be given the general form:

$$t_x = K \frac{a}{b} \quad (4)$$

where x = per cent of nominal concentration attained in time t , and K = a constant.

Values of K for various values of x are

x	K
99	4.605
95	2.996
90	2.303
85	1.897
80	1.609

Experimental verification of this principle is presented as follows.

In one series of experiments, chlorine was introduced at a constant rate into a 629 liter chamber, operated at three different constant air-flow levels, 125, 250, and 500 liters per minute. Samples were drawn at regular intervals and analyzed until three analyses coincided. In Table I a comparison is made between these analytical concentrations, expressed as percentages of the final

TABLE I. TIME TO REACH CONSTANT CONCENTRATION IN 629 LITER CHAMBER

CHAMBER AIR FLOW (LITERS PER MINUTE)	TIME (MINUTES)	PER CENT FINAL CONCENTRATION	
		OBSERVED	CALCULATED
125	2	32	33
	4	56	55
	6	70	70
	8	78	80
	10	84	86
	12	89	91
	14	90	94
	16	99.5	96
	18	100	97
	20	100	98
	22	100	99
250	2	56	55
	3	71	70
	4	80	80
	5	90	86
	6	90	91
	7	94	94
	8	97	96
	9	99	97
	10	99	98
	11	100	99
	12	100	99.2
	13	100	99.4
500	2	80	80
	3	92	91
	4	96	96
	5	99	98
	6	100	99.2
	7	100	99.6
	8	100	99.8

concentration, and the theoretical per cent of the constant value concentration, which should be reached at the time of sampling, as predicted by the use of Equation 2. The results, given in Table I, showed a close agreement between the observed and calculated values.

In another series of experiments, dichlorodiethyl sulfide was dispersed into a 20,000 liter chamber which was operated at an air flow of 3,600 liters per minute in Table II, the actual per cent of the final concentrations found by sampling at regular intervals are compared with calculated values, as in Table I. The observed and calculated percentages also showed close agreement.

TABLE II. TIME TO REACH CONSTANT CONCENTRATION IN 20,000 LITER CHAMBER;
AIR FLOW, 3,600 LITERS PER MINUTE

TIME (MINUTES)	PER CENT FINAL CONCENTRATION	
	FOUND	CALCULATED
2	33	30
5	57	59
8	77	76
11	86	86
14	93	92
17	97	95
20	97	97
23	100	99

It may be seen from Equation 3 that t_{90} is independent of any previous concentration level in the chamber. Therefore, for any fixed chamber volume and air flow, the time required for a change from one state of equilibration to another is always the same. It is thus obvious that t_{90} , for clearing the chamber after stopping the flow of agent, is identical with the t_{90} for establishing the concentration. Moreover, if the concentration in the chamber is changed after equilibration, the time for attaining the new level also is the same as the equilibration time.

For low air flows and high sampling rates, the rate of sample collection should be added as a correction to the chamber air flow for the most accurate calculations of equilibration time. However, when the sampling rate is less than 1 per cent of the air flow, this correction may be considered negligible and can be omitted.

Ideally, after equilibration, the analytical concentration should approximate the nominal concentration. Actually, perhaps because of surface effects within the chamber, there may be a significant discrepancy.

SURFACE EFFECTS

The interior surface of the chamber to which the agent is exposed may lower its concentration because of absorption, adsorption, or chemical reaction. An illustration of surface effects is given by the following experimental data. Concentrations of chlorovinyl dichloroarsine were established in a 386 liter chamber operated at 250 liters per minute. After equilibration, samples were collected and analyzed by iodometric titration. In one series, the interior surfaces of the chamber were coated with a cellulose acetate lacquer. In another

series, the surfaces were covered with a "corrosion-proof" lacquer.* The results are given in Table III, which shows that the cellulose acetate coating caused a much greater decrease in concentration than did the "corrosion-proof" lacquer.

TABLE III. EFFECT OF TYPE OF SURFACE ON CHAMBER CONCENTRATION AGENT, CHLOROVINYL DICHLOROARSINE

SURFACE	NUMBER OF EXPERIMENTS	AVERAGE NOMINAL CONCENTRATION FOUND (PER CENT)	S.D.
Cellulose acetate	8	13	± 9
Cotoid	39	93	± 5

This effect may be minimized by selection of the proper surface for each agent to be used in the chamber. Thus, the interior may be glass- or enamel-lined or lacquered. For most compounds, a corrosion-resistant lacquer is sufficient to prevent appreciable loss of concentration. However, it should be emphasized that the presence of even small surfaces of rubber will decrease the concentrations of certain organic vapors to a marked degree. For example, a 1 foot section of $\frac{1}{4}$ inch rubber tubing in the sampling line completely absorbed the chlorovinyl dichloroarsine in a 10 liter sample containing 0.25 mg. per liter. The same sized section of rubber tubing thrown into a gassing chamber reduced the concentration of the same compound in the chamber by about 20 to 30 per cent. Similar effects of rubber have been noted with dichlorodiethyl sulfide and other organic sulfides. In this laboratory, all rubber surfaces in the chamber and sample collection line are either coated with lacquer or are eliminated by using either ground glass joints or glass to glass connections in assembling sampling lines. Inasmuch as the type and extent of interaction of surface upon any particular agent cannot be predicted, it is necessary to make analytical-nominal concentration comparisons to ascertain this effect before any experimental gassing is performed.

The magnitude of any surface effect depends on the quantity of surface exposed per unit volume and is governed by the shape and size of the chamber and the presence of extraneous interior surface. The surface/volume ratio is a function largely of the size and shape of the chamber. A spherical chamber has the smallest surface/volume ratio. However, a cubical chamber is more easily constructed, and since it has a lower surface/volume ratio than the other rectangular shapes, it is the preferred form. Since the surface/volume ratio of either type of chamber is inversely proportional to the cube root of the volume, it decreases as chamber size increases. Obviously, protuberances and extraneous apparatus or accessories such as fans and lights, within the chamber, which increase the surface/volume ratio should be avoided wherever possible.

Fans, often used as a means of promoting even distribution of agent in the chamber, have been found unnecessary in this laboratory. The air flow itself tends to produce a completely satisfactory distribution, even in the absence of mixing bowls or other premixing devices, as shown by the following data taken from work of Wells.¹²

*Cotoid, Lithgow Corporation, Chicago, Ill.

A concentration of chlorine was established in a 629 liter cubic chamber operated at an air flow of 500 liters per minute. Samples were taken from nine locations, identified by number, within the chamber. Sampling point 1 was the center of the chamber, while the other eight points were the eight corners. A series of experiments was performed in which samples were drawn simultaneously from point 1 and one other point, after equilibration. Concentrations were calculated from the results of iodometric analysis. It was found that the concentration in the corners was the same as that in the center of the chamber for any given sampling time. The results are given in Table IV.

TABLE IV. DISTRIBUTION OF CHLORINE IN CONSTANT FLOW CHAMBER

RUN NO.	SAMPLING POINTS	CONCENTRATION CHLORINE FOUND (MILLIGRAMS PER LITER)
1	1	0.97
	2	0.96
2	1	0.94
	2	0.94
3	1	0.97
	3	0.98
4	1	0.98
	3	0.97
5	1	0.97
	4	0.97
6	1	0.98
	4	0.97
7	1	0.97
	5	0.97
8	1	0.98
	5	0.98
9	1	0.97
	6	0.97
10	1	0.98
	6	0.98
11	1	0.97
	7	0.97
12	1	0.97
	7	0.97
13	1	0.97
	8	0.97
14	1	0.97
	8	0.97
15	1	0.97
	9	0.96
16	1	0.97
	9	0.97

RELATION OF DOOR SIZE TO AIR INLET

Concentration losses may occur if the chamber air inlet is too small in relation to the size of the door.

When the door of a gassing chamber is opened, air enters through the door as well as through the inlet. The proportion of the total air entering the chamber through these openings is governed by their proportional cross sectional areas and may be calculated.

One of the small animal chambers in this laboratory has a circular air inlet 2 inches in diameter (cross-sectional area = 3.1 square inches) and a square door measuring 7 by 7 inches (cross-sectional area = 49 square inches). When

the door is opened for inserting or withdrawing animals, 6 per cent of the chamber air flow enters the inlet, and 94 per cent enters the door opening. This particular chamber is usually operated at an air flow of 250 liters per minute. Thus, during the time the door is open, 235 liters per minute enter the door and 15 liters per minute enter the inlet. If an agent is being vaporized by means of air or inert gas, or is being dispersed in the gaseous state, the agent flow is limited to 15 liters per minute. Any flow in excess of that is not drawn into the chamber but is blown back into the room.

Although in actual operation the door of the chamber remains open only a brief period of time, this factor has an important influence on chamber behavior. Since the time required to restore the concentration to its previous level is the same as that for the original equilibration, a significant decrease in chamber concentration may cause serious errors for short exposure periods.

To promote turbulence and good mixing in the chamber, the agent inlet should be small enough to insure a rather high rate of linear flow. This limits the door size to a maximum cross sectional area determined by the desired agent flow and the chamber air flow. With higher rates of air flow, larger doors may be permitted, but the ease of setting up concentrations is thereby decreased because of the attendant necessary increase in rate of agent volatilization.

EFFECT OF ANIMALS OR SUBJECTS

Animals or subjects within a chamber have an effect upon the concentration because of the additional surface presented. This is demonstrated by the following experiments.

Clothed and naked men were exposed to various established concentrations of dichlorodiethyl sulfide vapor in a 20,000 liter chamber operated at an air flow of 3,600 liters per minute. The entry of the men into the chamber caused an almost immediate drop in the concentration which then remained for the duration of the exposure (from 10 to 120 minutes) at its new lowered level. The concentration drop was much more pronounced with clothed men than with naked men because of differences in the nature and quantity of the added surface. The data are presented in Table V.

TABLE V. CONCENTRATION DROP DUE TO SURFACE OF SUBJECTS

DRESS	NUMBER OF SUBJECTS	NUMBER OF EXPERIMENTS	AVERAGE CONCENTRATION DROP (PER CENT)	CONCENTRATION DROP PER SUBJECT (PER CENT)
Clothed	4	10	22.3	5.6
Clothed	5	29	26.7	5.3
Naked	5	13	8.0	1.6
Naked	8	10	11.8	1.5

TABLE VI. CONCENTRATION DROP DUE TO SURFACE OF ANIMALS

NUMBER OF MICE	NUMBER OF EXPERIMENTS	AVERAGE CONCENTRATION DROP (PER CENT)	CONCENTRATION DROP PER MOUSE (PER CENT)
20	26	17	0.9
10	2	10	1.0

In another series of experiments, placing mice in a 386 liter chamber operated at an air flow of 250 liters per minute, produced a drop in the previously established chamber concentration of chlorovinyl dichlorarsine. The results are shown in Table VI.

To minimize surface effects due to animals, the volume of the chamber should be large compared with the volume of the animals exposed. It has been found in this laboratory that excessive concentration lowerings occur when the volume of the animals is more than 5 per cent of the chamber volume.

The rate at which animals or subjects enter the chamber may also cause a drop in the concentration. The volume of animal or subject entering per unit time must be less than the volume of air entering the door, if displacement of chamber air into the laboratory is to be avoided.

Some examples will illustrate this point. In a large chamber used in this laboratory for human exposures, at an air flow of 4,000 liters per minute, 98.3 per cent or 3,932 liters per minute enter through the door when it is opened. This is equivalent to 65.5 liters per second. An average man, having a volume of about 70 liters, would therefore enter in not less than 1.07 seconds. In usual practice, about 1.5 seconds are allowed for each man entering the chamber.

A smaller chamber used for exposures of small animals is usually operated at an air flow of 250 liters per minute. When the door is opened, 4 liters per second enter the door. Twenty mice, occupying a volume under 0.5 liter, can be inserted in as quickly as 0.125 second without displacing the air. However, six rabbits, with a volume of about 12 to 15 liters, must be introduced over a period of from three to four seconds.

DISCUSSION

Serious errors in gassing experiments may be made if exposures are initiated before equilibration. Probably the greatest contributor to such errors is a misinterpretation of the term "air change." An "air change" is said to occur when a volume of air equal to the volume of the chamber has passed through the chamber. However, one "air change" does not completely renew the chamber air, but, from Equation 2, should change the concentration within by only 63.2 per cent. From Equation 3 it is obvious that the time for one "air change" must be multiplied by a factor of 4.605 before even 99 per cent of the expected change can be accomplished. The term "air change" is therefore misleading, especially to the uninitiated, and should be eliminated from gassing terminology.

It is hoped that this exposition will be of help to those who have noted apparently irregular behavior in gassing chambers. A knowledge of the principles previously discussed should clarify many of these anomalies. The way in which concentrations build up has been discussed, and emphasis is to be placed upon the importance of the equilibration time. However, the effects of other factors on concentrations after equilibration cannot be neglected and may cause considerable deviations from calculated results. It is therefore necessary that regular analyses of chamber concentrations be made for the constant information of the operator.

SUMMARY

The principles discussed in this paper may be summarized as follows:

1. The *air flow* through the chamber influences:
 - (a) The time for equilibration to 99 per cent of theoretically constant concentration.
 - (b) The concentration which can be established for any given agent.
 - (c) The speed at which animals or subjects may be inserted.
2. The *size* of the chamber influences:
 - (a) The time for equilibration.
 - (b) The loss in concentration due to surface effects.
 - (c) The number of animals or subjects which can be exposed at one time.
3. The *character and quantity of the interior surface* of the chamber influence the loss in concentration due to surface effects.
4. The *shape* of the chamber influences the surface/volume relationship, and thus the loss in concentration due to surface effects.
5. The *relative areas of air inlet and door opening* influence the maximum rate of agent dispersal.
6. The *number and size of the animals* influence:
 - (a) The rate of insertion of animals into the chamber.
 - (b) The loss in concentration due to surface effects.

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THE SIMPLE ESTIMATION OF BLOOD KETONES IN DIABETIC ACIDOSIS

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IT CANNOT be too strongly emphasized that diabetic acidosis demands early diagnosis and prompt treatment. Occasions arise when clinical appraisal of the patient is inadequate for positive diagnosis. Emergency determinations of blood glucose and carbon dioxide combining power are sometimes employed. A high blood sugar level, however, is not diagnostic of acidosis, and a reliable determination of CO_2 is not always obtainable in an emergency. Because there is a parallel between the blood ketone level and the severity of diabetic acidosis, a simple clinical method for the estimation of blood ketones would be of distinct value.

Routinely, the urine is tested for acetone and diacetic acid. Several authors have suggested the use of a dry powder for the qualitative detection of these compounds.¹⁻³ A drop of urine added to the powder gives a prompt and distinctive purple color when acetone is present.* There are a number of diabetic patients whose disease is severe and who frequently show large amounts of sugar in the urine. Under the influence of a slight infection, these patients may rapidly develop a diabetic acidosis. Since the early detection of ketonuria, when followed by prompt therapy, will do much to prevent the onset of clinical acidosis, we have found it worth while to provide the patients with more severe diabetes in our clinic with this powder. The test is simple and inexpensive and need only be used when the routine test for sugar indicates excessive glycosuria.

We have studied the application of this test to the determination of blood ketones, and it has been found that the same color reaction will appear when serum of a high ketone content is added to the powder. The minimal blood level of total acetone bodies giving a definitely positive test is approximately 10 mg. per 100 c.c. as determined by the method of Shipley and Long.⁴ Thus, the test can quickly be made quantitative by the successive dilution of serum with distilled water. The last dilution to give a positive reaction, when multiplied by ten, will give the blood ketone level within an accuracy of plus or minus 10 mg. per 100 c.c. For example, if the undiluted serum has been shown to give a positive test, then 1 c.c. is mixed with an equal amount of water. If a positive reaction is still present, additional portions of water are added until the test becomes negative. In a given sample, the last positive dilution might contain 1 c.c. of serum and 6 c.c. of water. This would represent a dilution of

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*Our own formula for the powder has been:

Sodium nitroprusside, 1 Gm. (very finely ground)

Ammonium sulfate, 20 Gm.

Anhydrous sodium carbonate, 20 Gm.

The three ingredients are mixed completely but are not ground together. The compound should be kept dry at all times. Stability has been shown to be complete for over one year.

The test is performed by placing a small pinch of the powder, 5 mm. in diameter, on a white filter paper. One drop of urine or serum gives a prompt violet color reaction which is absorbed into the paper and which may last for several hours.

A powder of somewhat similar composition is available commercially.

1 part in 7, and the result would be approximately 70 mg. per 100 c.c. If only one drop is removed after each succeeding dilution, the error introduced by withdrawal of the test sample will be less than 5 per cent.

The value and relative accuracy of this simple test have been shown in the diagnosis and treatment of diabetic coma at Lakeside Hospital. It has been the practice recently to follow the blood ketone levels during active therapy in diabetic acidosis. In some twenty-five instances, the values of the simple spot test have been shown to correspond to the more accurate simultaneous micro determinations with an error seldom exceeding 10 mg. per 100 c.c. The range covered has been from 0 to 100 mg. per 100 c.c. It is true that the micro test determines total ketone bodies while the test with the powder measures only acetone and diacetic acid, yet the numerical values have been valid in the range mentioned. This accuracy has been so consistent that it has become routine to use the test when there is any doubt as to the severity of ketosis. By virtue of its rapidity and simplicity, it has proved more valuable than the emergency determinations of CO_2 combining power in providing immediate laboratory confirmation.

In the diagnosis and treatment of diabetic acidosis, the following seems to be worth emphasis. With a typical history and findings, diagnosis presents no problem. Often, however, the history is not typical. More important, the presence or absence of acetone in the urine may not indicate the actual blood ketone level. It has been pointed out that in renal failure, ketonuria may be minimal in spite of severe ketonemia. Furthermore, in patients without renal failure but with diminished kidney function, the threshold for ketone excretion may be abnormally high. Thus, the previously mentioned test which measures blood ketone levels will be considerably more useful than a test for acetone in the urine. It has also been valuable in quickly separating true diabetic acidosis from those cases in which a poorly controlled diabetes has been accompanied by a surgical abdomen, by vomiting, or by coma from other causes and in which blood ketones have not been high. In these latter cases, the vigorous therapy demanded in true diabetic acidosis is not needed, and the necessary surgical therapy can proceed promptly. Ketone levels in these cases seldom exceed 20 mg. per 100 c.c., while those of diabetic acidosis are invariably over 50 mg. per 100 c.c.

SUMMARY

Acetone bodies in serum may be estimated within an error usually not exceeding 10 mg. per cent by the use of a simple technique employing a nitroprusside powder. The method is particularly useful for the prompt differentiation of minimal ketonuria due to minimal ketonemia, as contrasted with cases of a high renal threshold resulting from depressed renal function in which minimal ketonuria may be associated with severe ketonemia.

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BOOK REVIEWS

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ANNOUNCEMENT

The first session of the Pathology Study Section of the National Institute of Health's Research Grants Division was held at the Institute in Bethesda, Md., on Aug. 16, 1946.

The Pathology Study Section, which is one of more than twenty groups of outstanding consultants advising in the whole field of medical research, is comprised of the following men: Dr. Paul Cannon, Chairman, University of Chicago, Chicago, Ill.; Dr. W. A. DeMonbreun, Vanderbilt University, Nashville, Tenn.; Dr. W. H. Feldman, Mayo Foundation, Rochester, Minn.; Dr. W. D. Forbus, Duke University, Durham, N. C.; Dr. H. Goldblatt, Cedars of Lebanon Hospital, Los Angeles, Calif.; Dr. J. S. McCartney, University of Minnesota, Minneapolis, Minn.; Dr. A. R. Moritz, Harvard University, Boston, Mass.; Dr. A. Rich, Johns Hopkins University, Baltimore, Md.; Dr. J. F. Rinehart, University of California, San Francisco, Calif.; Dr. H. P. Smith, Columbia University, New York, N. Y.; representatives of the Surgeons General of the United States Army and United States Navy, members of the Medical Department of the Veterans' Administration, and Dr. R. D. Lillie, National Institute of Health, Bethesda, Md., as Executive Secretary.

A number of projects were considered and recommendations made to the National Advisory Health Council for final action. The Section members agreed unanimously that individually they would promote necessary research in the field of pathology by private suggestions but would not make any collective attempts to impose research projects on investigators or institutions. They also approved the attitude of the Public Health Service that the scientific freedom of the research investigators must not be restricted in any way.

Application forms for Grants-in-Aid may be obtained from the Chief, Research Grants Division, National Institute of Health, Bethesda 14, Md., and for prompt action should be filed well in advance of the quarterly meetings of the Study Section concerned.

The next meeting of the Pathology Study Section was scheduled for an early date in November, 1946.

SUBCUTANEOUS VS. INTRAMUSCULAR ADMINISTRATION OF PENICILLIN

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FOR the parenteral administration of penicillin, the intramuscular route has been recommended as the most practical one when intermittent injections are given.¹ Intravenous injections, though not so painful, are less desirable because they do not produce a sufficiently sustained plasma concentration of penicillin. On the other hand, subcutaneous injections, which should at least theoretically give more sustained plasma levels than either intramuscular or intravenous injections, have been viewed with disfavor because of the irritation produced. Accordingly, the most recent directive of the War Production Board² has stated that the subcutaneous route should be avoided.

These recommendations have been based on the properties of the penicillin commercially available at the time. That the degree of purity of the penicillin is the important factor in determining the intensity and duration of the pain produced by the injections has been shown by Herwick and co-workers.³ Now that pure or almost pure preparations of penicillin have been made available for clinical use, the question of the most desirable route for parenteral administration should be re-examined. The results of such an investigation are summarized in this report.

METHODS

The following penicillin preparations were used for this study:

1. An almost pure amorphous sodium penicillin (1,550 units per milligram) put up in 200,000 unit vials. This will be spoken of as high potency sodium penicillin (amorphous).
2. Crystalline sodium penicillin (1,400 units per milligram) in 200,000 unit vials.
3. Pure crystalline potassium penicillin (1,631 units per milligram) in 100,000 unit vials. (An earlier amorphous preparation used in a few instances contained 1,501 units per milligram.)*

The penicillin was dissolved in sterile physiologic saline solution in a concentration of 10,000 units per cubic centimeter. When injected intramuscularly, the triceps or deltoid muscle was utilized, a 1½ inch, 22 gauge needle being

From the Hektoen Institute for Medical Research of the Cook County Hospital.

Aided by a grant from the Commercial Solvents Corporation, Terre Haute, Ind.

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*These products were kindly supplied by the Commercial Solvents Corporation, Terre Haute, Ind. According to the producers, chromatographic data, differential assays, and other tests indicate that over 90 per cent of the penicillin present in all three of these preparations is Penicillin G.

employed. For subcutaneous injections, the upper arms or thighs were chosen, and the regular $\frac{1}{2}$ inch, 26 gauge hypodermic needles were employed.

The penicillin preparations were injected subcutaneously or intramuscularly in doses of 10,000, 15,000, or 20,000 units into hospital control subjects who were young or middle-aged patients, not acutely ill, and who had no demonstrable circulatory or renal disturbances. Plasma penicillin concentrations were determined at one-half, two and one-half, and three hours after the injections by the serial dilution *Bacillus subtilis* method of Randall, Price, and Welch⁴ as modified by Hickey.⁵ In the majority of injections of penicillin, the subcutaneous and intramuscular comparisons were made in the same patient; however, since there was a great degree of variability of the plasma levels achieved with any penicillin injection even in the same patient by the same route, the results have been evaluated by the determination of the average of plasma values for any type of injection.

In order to determine whether human blood, even in the absence of penicillin, has inhibitors of growth of *B. subtilis*, as has been alleged,⁶ control blood samples were taken for penicillin estimation before penicillin was administered in forty assays. In addition, blood specimens from eighty hospital patients not on penicillin or sulfonamide therapy were analyzed for penicillin. In the total of 120 plasma specimens, only one showed a level equivalent to 0.03 unit; the remainder gave 0 levels. These findings, supplemented by the many 0 levels in the hundreds of assays made during these and other studies, have convinced us that, at least with our use of the method and possibly because of the employment of citrated plasma, there is little danger of finding positive penicillin levels in plasma specimens that do not contain penicillin.

RESULTS

Pain.—Preliminary experiments have already demonstrated that with none of the three preparations was pain an important factor for either the subcutaneous or the intramuscular route. When any of these three preparations was given alternately for therapeutic purposes with the common impure commercial preparations (six different brands commonly purchased by the hospital), the patient had no difficulty distinguishing between the relatively pure and the low potency brands, even though they did not see the solutions injected. The purified material produced only the pain of injection; there was no prolonged pain. However, the impure yellow preparations gave a "bee-sting" type of irritation that lasted from three to fifteen minutes. This finding was confirmed by my personal experience. Similarly, in all the assays the subjects did not complain of any prolonged pain following either subcutaneous or intramuscular injection. In fact, the subcutaneous injections, made as they were with a small needle, were for the most part regarded as less annoying. It is particularly worth noting that the potassium penicillin, which previously had never been used in any extensive series of injections, produced no more pain or other reactions than did the two sodium preparations. Also, in this series of 206 assays, there were no recognized cases of urticaria or other allergic manifestation, which finding may be related to the purity of the preparation used.

TABLE I. FREQUENCY DISTRIBUTIONS OF PLASMA PENICILLIN LEVELS AFTER SUBCUTANEOUS OR INTRAMUSCULAR INJECTIONS OF PURIFIED PENICILLIN PREPARATIONS

DOSE (UNITS)	PLASMA PENICIL- LIN CONCEN- TRATION (UNITS PER C.C.)	HIGH POTENCY SODIUM PENICILLIN (AMORPHOUS)						POTASSIUM PENICILLIN						CRYSTALLINE SODIUM PENICILLIN					
		SUBCU- TANEOUS HOUR			INTRAMUS- CULAR HOUR			SUBCU- TANEOUS HOUR			INTRAMUS- CULAR HOUR			SUBCU- TANEOUS HOUR			INTRAMUS- CULAR HOUR		
		1	2½	3	1	2½	3	1	2½	3	1	2½	3	1	2½	3	1	2½	3
10,000	1.0																		
	0.5	3			3			1			1			1			1		
	0.25	8			6			8			8			7			7		
	0.125	1	1		3			1			1	1		2			2		
	0.062		3			7	2		1		3	2						1	1
	0.031		6	4		2	2		9	6		6	5		8	7		7	5
	0		2	8		3	8			4		3			2	3		2	4
15,000	1.0																		
	0.5	3			1			5			5			4			3		
	0.25	6			6			3			5	1		13			6		
	0.125	1			3	1		2			1			1			1		
	0.06		3	2		1	3	3	3	1	5	3		6	3			4	2
	0.03		5	4		6	3		4	4	3	3		8	10			5	5
	0		2	3		2	4		2	6		4		4	5			1	3
20,000	1.0	2			1			1			3			3			1		
	0.5	8			12			6			3			5			8		
	0.25	5	1		2			3			4			2	1		3		
	0.125		6	1		7					1					1		2	
	0.062		5	7		5	2		6	2	5	2		6	1			8	5
	0.031		3	5		2	8		3	6	4	6		2	6			2	5
	0			2		1	5		1	2		2		1	2				2

Comparison of Plasma Penicillin Levels.—The frequency distribution of plasma Penicillin levels at one-half hour, two and one-half hours, and three hours after subcutaneous or intramuscular injections of 10,000, 15,000, or 20,000 units of penicillin is shown in Table I. A total of 206 assays were made involving 618 determinations of plasma penicillin concentrations. It will be seen that there was a considerable variation in the levels obtained for any one type of injection in the several subjects but that usually the modal level could be easily determined. In general, the plasma level for 10,000 units by either subcutaneous or intramuscular injections at one-half hour was 0.25 unit per cubic centimeter; at two and one-half hours, 0.03 unit per cubic centimeter; and at three hours, 0 unit per cubic centimeter (that is less than 0.03). For 15,000 units, there was no great difference at one-half hour or at two and one-half hours, but at three hours the prevailing level was 0.03 unit per cubic centimeter. For 20,000 units, the modal value was 0.50 unit per cubic centimeter at one-half hour, 0.06 unit per cubic centimeter at two and one-half hours, and 0.03 unit per cubic centimeter at three hours.

For a more rigid comparison of the levels obtainable after the subcutaneous and intramuscular injections of the different dosages of the different preparations, the mean levels were tabulated in Table II. It should be recognized that the use of the arithmetic mean in averaging values that progress geometrically is likely to give too much weight to isolated high values and that it might be better to obtain the average by giving an arithmetically progressing weighting to each level or otherwise to use the geometrical mean.

TABLE II. AVERAGE PLASMA PENICILLIN LEVELS AFTER SUBCUTANEOUS OR INTRAMUSCULAR INJECTION OF PURIFIED PENICILLIN PREPARATIONS

DOSE (UNITS)	TYPE OF INJECTION	HIGH POTENCY SODIUM (AMORPHOUS)			CRYSTALLINE SODIUM			CRYSTALLINE POTASSIUM			TOTAL AVERAGE		
		1 HR.	2½ HR.	3 HR.	1 HR.	2½ HR.	3 HR.	1 HR.	2½ HR.	3 HR.	1 HR.	2½ HR.	3 HR.
10,000	Subcutaneous	.302	.012	.010	.263	.034	.019	.250	.025	.022	.272	.034	.017
	Intramuscular	.281	.042	.015	.263	.050	.028	.250	.029	.022	.265	.040	.022
15,000	Subcutaneous	.313	.035	.025	.312	.056	.016	.300	.035	.028	.308	.042	.023
	Intramuscular	.238	.037	.028	.375	.078	.028	.313	.041	.028	.308	.052	.028
20,000	Subcutaneous	.483	.094	.018	.475	.047	.031	.600	.079	.038	.519	.070	.039
	Intramuscular	.500	.065	.025	.550	.056	.031	.480	.068	.047	.510	.063	.034

Nevertheless, in spite of the handicap of the method, it could readily be seen that there was no appreciable difference between the levels obtained after subcutaneous injections and those after intramuscular injections. It could also be recognized that all three preparations, the two sodium penicillins and the potassium, gave essentially the same results. The average levels for each interval for all the injections (see Table II) agree fairly closely with the modal values previously mentioned. The plasma levels found indicate that, even with such a low dosage as 10,000 units of these purified preparations every three hours, there is likely to be therapeutic levels during at least two and one-half hours and that the chief value of giving 15,000 or 20,000 units is to assure a therapeutic level in the last one-half hour or hour. A dosage of 15,000 units of purified penicillin every three hours would probably be effective in all infections by bacteria relatively sensitive to penicillin, which include the gonococcus, pneumococcus, *Staphylococcus aureus*, and meningococcus. On the other hand, in cases of infections by more resistant organisms, such as the *Staphylococcus albus* and *Streptococcus viridans*, where plasma levels of the order of 0.5 unit per cubic centimeter or higher may be required, dosages of 20,000 units every three hours are not high enough to insure therapeutic levels.

That subcutaneous injections of purified penicillin gave no more prolonged levels than did intramuscular injections may be due to the relative insensitivity of the serial dilution method used for the assay of penicillin. Nevertheless, producing, as it does, at least as good and prolonged levels as intramuscular injections, the subcutaneous route becomes the method of parenteral administration of choice.

SUMMARY

Comparison of subcutaneous and intramuscular injections of three purified preparations of penicillin were made in 206 assays involving 618 plasma penicillin level estimations.

There was no afterpain with either type of injection. Subcutaneous injections, because they permitted the use of a small needle, were less annoying than those given intramuscularly.

There were no essential differences between the plasma penicillin levels obtained after subcutaneous injections and those following intramuscular ad-

ministration. Also, all three purified preparations, the amorphous and the crystalline sodium penicillin and the crystalline potassium penicillin, gave essentially the same results.

There were no instances of urticaria or other forms of sensitivity in all 206 assays.

The subcutaneous route has been found from our studies to be the one of choice for the parenteral administration of purified penicillin preparations.

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PERMEABILITY ALTERATIONS IN DISEASE

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IN THE course of recent investigations into the fluid and ionic balance in simian malaria,¹ a large number of determinations of "available fluid" (extracellular) volumes were done by the sodium thiocyanate method² (1) in normal monkeys, (2) in monkeys with fatal *Plasmodium knowlesi* infections, and (3) in monkeys with malaria which were treated subsequently with sulfadiazine and survived. In twenty-three monkeys control thiocyanate volumes ranged from 16.6 to 28.8 per cent of the body weight with a mean value of 20.8 per cent. The spread in these values is less if measurements obtained in the summer or winter are considered separately, for the monkey exhibits a larger extracellular volume in summer. There appears to be a similar seasonal variation in blood, plasma, and extracellular volumes in man.³

Following the induction of *P. knowlesi* infection in monkeys the volume of fluid available for dilution of NaSCN increased in a progressive manner throughout the course of the disease. This progressive change in two animals (5AS and 6AS) and the return toward normal following chemotherapeutic intervention is shown in Fig. 1. At first it was thought that water shifts were occurring and that the extracellular compartment was becoming superhydrated. However, as the parasitemic level rose, the apparent extracellular fluid volume increased to such a degree that it became obvious that the changes could not be due to water shifts alone. In all cases of malaria fatalities in which measurements were made within twenty-four hours preceding death, the volume of fluid available for dilution of thiocyanate increased until it exceeded 50 per cent of the body weight. A few animals in which determinations were made within the six-hour period preceding death revealed available fluid volumes of 64 to 66 per cent of the body weight. Furthermore, these volume changes occurred over a period of time in which plasma volumes fell slightly and the animals lost weight.

Harrison, Darrow, and Yannet,⁴ employing the desiccation technique, found that the total body water of the monkey is approximately 65 per cent of the body weight. It is obvious at once that NaSCN determinations do not, in the malarious animal, represent the extracellular volume.

At autopsy, neither the peritoneal cavity nor the intestine exhibited any gross accumulation of fluid. Cut surfaces of all organs appeared dry and dehydrated. Further, the time-concentration curves of NaSCN in plasma were of no steeper slope than in normal animals, indicating that NaSCN was neither being destroyed nor lost from the body at an increased rate.

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In five animals which died of malaria, tissue thiocyanate determinations were made immediately after death on heart, liver, spleen, and muscle. The amount of thiocyanate found in these tissues varied, liver having the highest and muscle the least (Table I). However, in all tissues, the amount found was larger than could have been resident in the chloride space of the tissue. In the course of simian malaria, at least, the dilution of sodium thiocyanate is no longer a measure of the extracellular fluid. The conclusion is inescapable that, in malaria, sodium thiocyanate enters the cells and becomes diluted by the intracellular water.

TABLE I. RESULTS OF TISSUE AND PLASMA THIOCYANATE DETERMINATIONS

ANIMAL	TISSUE	SCN IN CL SPACE OF 1,000 GM. TISSUE*	SCN FOUND IN 1,000 GM. TISSUE	SCN IN 1,000 C.C. INTRACELLU- LAR WATER	SCN SPACE AS PER CENT BODY WEIGHT
		(MG.)	(MG.)	(MG.)	
9AT	Muscle	4.82	5.1	0.28	28.8
11AK	Muscle	3.98	5.1	1.12	33.1
7AK	Muscle	2.98	5.0	2.01	39.8
8AK	Muscle	2.16	5.2	2.04	48.2
10AT	Muscle	1.81	5.1	3.29	54.6
9AT	Liver	13.49	16.32	2.83	28.8
11AK	Liver	11.17	22.44	11.27	33.1
7AK	Liver	8.37	15.0	6.63	39.8
8AK	Liver	6.04	15.6	9.56	48.2
10AT	Liver	5.07	17.34	12.27	54.6
9AT	Spleen	13.10	10.20	none	28.8
11AK	Spleen	10.54	18.36	7.52	33.1
7AK	Spleen	8.13	7.0	none	39.8
8AK	Spleen	5.87	8.84	2.97	48.2
10AT	Spleen	4.93	22.44	17.51	54.6
9AT	Plasma	35.41	31.90		28.8
11AK	Plasma	29.30	26.40		33.1
7AK	Plasma	21.98	19.80		39.8
8AK	Plasma	15.87	14.30		48.2
10AT	Plasma	13.32	12.00		54.6

*Calculated from the plasma level of SCN and Lepore's³ values for the chloride space of muscle, liver, and spleen.

That such a permeability change may be a general phenomenon accompanying febrile diseases is suggested by the following findings: (1) Repeated injections of typhoid vaccine in dogs over a period of ten days effect a significant increase in apparent extracellular volume.⁵ (2) Measurements made by Ling⁶ on patients with neglected compound fractures who had developed bacteremias with concomitant temperature elevations showed increases in the volume of fluid available for the dilution of NaSCN of the same order of magnitude as is being reported here in simian malaria. (3) NaSCN volumes measured by Harrel⁷ in patients with spotted fever likewise have indicated permeability alterations.

Although the survival time of Ling's patients did not allow repeated determinations of the NaSCN volume, the recovery phase of this permeability alteration has been studied in one group of the monkeys reported here and has been noted by Harrel in human patients with spotted fever. In the monkey the recovery period is long, normal permeability not being completely re-estab-

lished until about 100 days following the disappearance of clinical malaria (Fig. 1). According to Harrel, however, the NaSCN volumes of spotted fever patients subjected to therapy quickly returned to normal.

The indication that, in malaria, the permeability of cellular membranes is altered to the extent of allowing free passage of a foreign ion (SCN), which, in the normal animal, is generally excluded from the cell water, introduces a possible new interpretation of previous studies on native ion concentrations in paludic blood.

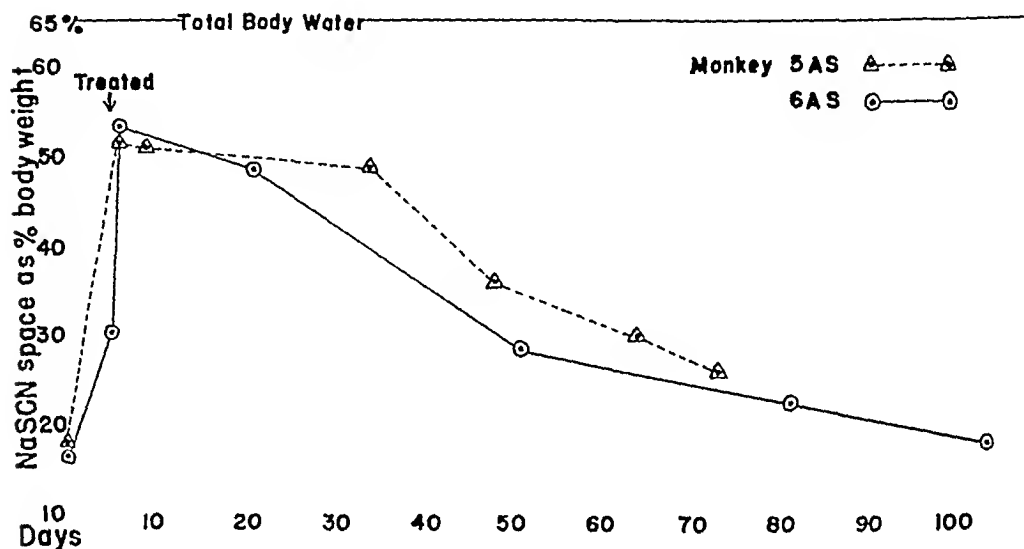


Fig. 1.

Pinelli,⁹ Velick and Seudder,¹⁰ Zwemer, Sims, and Coggeshal,¹¹ and Flosi¹² have all reported a decrease in blood sodium and/or an increase in blood potassium concentrations in malaria. Attempts have been made to correlate these findings with red cell destruction. Edelman and associates¹³ have described similar ionic shifts occurring in the blood of patients subjected to pyrexia induced by physical means alone (hyperthermia). The logical explanation of all of these results lies in the demonstration of generalized permeability alterations in the pyrexia state.

SUMMARY

1. The extracellular fluid volume as measured by the dilution of NaSCN increases in (1) simian malaria, (2) patients with spotted fever, and (3) patients with bacteremia who have concomitant temperature elevations.

2. Extracellular fluid volumes in these cases may increase to the point of being equal to the calculated total body water.

3. Tissue NaSCN determinations made immediately after death in monkeys in which *P. knowlesi* infections were fatal show larger amounts of NaSCN than could be resident in the "chloride space" of the tissue.

4. In these diseases, at least, the permeability of the tissue cells is altered, allowing the foreign ion (SCN) to become diluted by the intracellular water.
5. Animals showing such permeability changes return to normal slowly following chemotherapeutic intervention.
6. It appears that permeability alterations accompany the pyrexia state.

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GLYCOCYAMINE ELIMINATION IN PATIENTS WITH MYASTHENIA GRAVIS

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MUSCLE function has been shown to improve in patients with myasthenia gravis after infusion of amino acids.¹ Certain amino acids (glycine, arginine, sarcosine) may form glycoeyamine by transamidination, and glycoeyamine is methylated to creatine.²⁻⁵ Glycoeyamine is continuously eliminated in the urine of human beings.⁷⁻⁸ A disturbance in the decomposition of the mentioned amino acids, if severe, would manifest itself in changes of the amount of glycoeyamine eliminated, especially if the organism is overloaded with glycine. A severe decrease in transmethylation would manifest itself as a delayed or decreased formation of creatine from glycoeyamine and an increased elimination of glycoeyamine, especially if the organism is overloaded with glycine.

To ascertain whether the observed effect of amino acids on muscle function in patients with myasthenia gravis was due to correction of a deficiency of amino acids involved in creatine formation, the amounts of glycoeyamine eliminated in the urine of patients with myasthenia gravis before and after administration of glycine were determined.

METHOD

The glycoeyamine elimination of twelve patients with myasthenia gravis and twenty control subjects was determined by a modified method of Weber.⁷⁻¹⁰ All subjects were hospitalized and kept on a 2,000 calorie mixed diet. The control subjects had either ruptured intervertebral discs or were patients with cardiac arrhythmias but well compensated. Urine was collected in separate samples for twenty-four hours and kept in an icebox. Fifteen grams of glycine were then administered orally in one dose, and the collection of urine was continued for forty-eight hours.

All urine samples were diluted to a specific gravity of 1.010. To 5 c.c. of urine 2.5 c.c. of water, 2.5 c.c. of 0.4 N hydrochloric acid, and 500 mg. of Lloyd's reagent were added. The mixture was shaken vigorously for three minutes, then centrifuged, and the residue rinsed with water, slightly acidified with sulfuric acid. The precipitate was suspended in 8 c.c. of water, treated with 500 mg. of barium hydroxide, shaken for three minutes, and centrifuged. The precipitate was washed twice with 2 c.c. of water. The three supernatant fluids were pooled, and the barium in the filtrate was precipitated with 40 per cent sulfuric acid. The mixture was centrifuged. The supernatant fluid was shaken for three minutes with 1 Gm. of basic lead carbonate and centrifuged. This

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TABLE I. SHORT SUMMARY OF CLINICAL STATE OF TWELVE PATIENTS WITH MYASTHENIA GRAVIS

NAME	SEX	AGE	SEVERITY OF MYASTHENIA GRAVIS*	DURATION (YEARS)	THYROIDOMY	X-RAY TREATMENT	SYMPTOMATOLOGY	NEOSTIGMINE (PROSTIGMINE BROMIDE) †	
								DOSE (MG.)	ACTIVITY ON MEDICATION
Go	F	43	4+	7	No	Yes	Bedridden, severe lid ptosis, diplopia, difficulty in chewing and swallowing, severe muscular fatigability	135 (By mouth)	Sits in chair for a few hours
Ri	F	21	4+	1	No	Yes	Bedridden, severe lid ptosis, diplopia, difficulty in chewing and swallowing, severe muscular fatigability	150 (By mouth) 5 (By injection)	Chews food, walks few steps
Ro	F	26	3+	13	No	Yes	Moderate lid ptosis, occasional diplopia, moderate difficulty in chewing, moderate muscular fatigability	90 (By mouth)	Walks one to two blocks
An	F	21	3+	3	No	No	Moderate lid ptosis, occasional diplopia, difficulty in breathing and swallowing, occasional difficulty in chewing, moderate muscular fatigability	150 (By mouth)	Walks, feels comfortable
Ku	M	24	2+	2	No	Yes	Moderate lid ptosis, severe diplopia, difficulty in swallowing and chewing, moderate muscular fatigability	60 (By mouth)	Walks, slight sports
Po	F	28	1+	8	No	No	Moderate lid ptosis, moderate muscular fatigability	120 (By mouth)	Housework
Sh	M	48	1+	5	No	No	Complete lid ptosis, moderate muscular fatigability	30 (By mouth)	Works, horseback riding
Sch	M	25	1+	5	Yes	No	Slight lid ptosis, mild muscular fatigability	15 to 30 (By mouth)	Works as an engineer
Mu	F	30	1+	9	No	No	Moderate lid ptosis, rare diplopia, mild muscular fatigability	15 to 90 (By mouth)	Works as a cashier
Co	M	37	1+	1	No	No	Moderate lid ptosis, moderate diplopia, moderate difficulty in chewing and swallowing, mild muscular fatigability	45 (By mouth)	Walks, some work
Vi	F	23	1+	8	No	No	Slight lid ptosis, mild muscular fatigability	15 to 45 (By mouth)	Walks, goes to parties
La	M	48	1+	17	No	No	Severe lid ptosis	15 (By mouth)	Works as a tailor

*Most severely ill patient, † plus; least severely ill patient, † plus.

†Hoffmann-La Roche, Inc., Nutley, N. J.

supernatant fluid was shaken for eight minutes with 1 Gm. permittit and centrifuged. To 5 c.c. of the supernatant fluid, 1 c.c. of 10 per cent sodium hydroxide solution was added, and the test tubes containing the aliquots of the solutions to be analyzed were placed in an ice water bath. In this bath were also the α -naphthol (0.04 per cent), urea (40 per cent), and hypobromite (0.66 c.c. of liquid bromine in 100 c.c. of 5 per cent sodium hydroxide) solutions. After ten minutes 1 c.c. of the α -naphthol solution was added to each test tube, the contents shaken, 1 c.c. of the chilled urea solution added, and the contents shaken again. After another five-minute interval 0.5 c.c. of the chilled hypobromite solution was added, the contents mixed by vigorous shaking, and the tube replaced in the ice bath. After twenty minutes the color was determined with a Klett-Summerson photoelectric colorimeter using filter No. 54.

Material.—A short description of the clinical state of the patients with myasthenia gravis is given in Table I.

RESULTS

The amounts of glycocyamine eliminated in the urine before and after administration of glycine are given in Table II. The men used as controls elimi-

TABLE II. ELIMINATION OF GLYCOCYAMINE IN PATIENTS WITH MYASTHENIA GRAVIS AND IN CONTROL SUBJECTS

SUBJECTS	SEX	GLYCOCYAMINE ELIMINATED DURING 2½ HOURS (MG.)			
		DAY BEFORE GLYCINE	GLYCINE DAY	DAY AFTER GLYCINE	DIFFERENCE
Controls					
Da	M	59.0	79.7	58.2	20.7
Sa	M	44.5	68.5	43.0	22.0
Go	M	35.7	59.5	37.4	23.8
Fa	M	53.7	81.6	54.1	27.9
We	M	59.1	85.4	58.7	26.3
Bi	M	61.6	86.0	60.3	24.4
La	M	52.3	70.7	50.9	18.4
Ve	M	47.8	67.0	50.6	19.2
No	M	45.0	71.2	47.3	26.2
Da	F	63.0	105.2	63.9	42.2
No	F	36.5	61.3	38.9	24.8
Ho	F	82.8	122.9	80.6	40.1
He	F	80.2	138.4	80.5	58.2
Bo	F	55.7	111.2	57.4	55.7
Bu	F	33.2	62.9	36.0	29.7
El	F	70.4	90.2	68.7	19.8
La	F	63.7	114.5	62.5	50.8
Mo	F	77.2	99.0	78.4	21.8
To	F	60.7	90.3	63.9	29.7
Patients					
Ku	M	34.9	57.8	33.7	22.9
Sch	M	45.1	63.5	46.8	18.4
Sh	M	55.5	79.2	53.4	23.7
Co	M	43.2	69.0	47.8	25.8
La	M	46.6	72.4	44.4	25.8
Go	F	35.5	67.1	37.3	31.6
Ro	F	72.1	138.5	70.2	66.4
Ri	F	45.3	68.5	45.8	23.2
An	F	55.4	82.2	60.5	26.8
Pe	F	81.4	111.8	80.3	30.4
Mu	F	70.9	115.5	73.0	44.6
Vi	F	65.4	99.6	61.8	34.2

nated from 35 to 61 mg. glycoeyamine daily and the women used as controls from 33 to 82 mg. per day. Patients with myasthenia gravis eliminated similar amounts of glycoeyamine daily. Oral administration of glycine increased the elimination of glycoeyamine from 18 to about 66 mg. in both control subjects and patients with myasthenia gravis. Patients with myasthenia gravis excreted the largest amounts of glycoeyamine from four to six hours after administration of glycine. This seems to be a delay as compared to the data of Borsook and associates⁵ who found that the greatest amount of glycoeyamine is eliminated two hours after administration of glycine. The four- to six-hour peak lays, however, well within the limits observed with the control patients used in the present experiments.

DISCUSSION

The preceding results suggest that patients with myasthenia gravis eliminate glycoeyamine in amounts similar to human controls. Furthermore, glycoeyamine elimination after administration of glycine is similar in amounts in patients with myasthenia gravis and in control subjects. In so far as it is possible to determine by the amount of glycoeyamine eliminated, the processes of transamidination and transmethylation are not disturbed in patients with myasthenia gravis.

It is likely that glycoeyamine is formed mainly in the kidney by transamidination and is methylated mainly in the liver to form creatine.⁶ Striated muscle, however, also contains minute amounts of glycoeyamine (between 3 to 6 mg. per 100 Gm. of fresh rat muscle).⁶ Since a local increase of glycoeyamine in the muscle may not be detectable by the amounts of glycoeyamine eliminated in the urine and since, according to a recently presented concept,¹¹ most of the symptomatology of myasthenia gravis can be due to a decreased acetylcholine synthesis, it was also ascertained whether glycoeyamine modifies the synthesis of acetylcholine *in vitro* and whether it may modify the excitability of striated muscle. Glycoeyamine is without effect on these processes. Glycoeyamine does not significantly modify acetylcholine synthesis (only a 10 to 15 per cent decrease could be demonstrated with concentrations as high as 1 Gm. per 100 c.c.); it does not modify the sensitivity of muscle to indirect stimulation and chemical stimuli (acetylcholine, potassium).¹² Therefore, even if glycoeyamine were to be present in increased amounts in the muscle and nerve of patients with myasthenia gravis, it would not be responsible for the severe fatigability of the muscles of these patients.

SUMMARY

From the amount of glycoeyamine eliminated (before and after overloading the body with glycine) there is no evidence of any defect in transamidination and transmethylation processes in patients with myasthenia gravis.

The authors wish to express their gratitude to Dr. Joseph P. Chandler, for his valuable suggestions.

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ERYTHROCYTE FRAGILITY IN ACUTE INFECTIOUS HEPATITIS

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THE high incidence of acute infectious hepatitis in the military services has afforded an excellent opportunity for the investigation of both clinical and laboratory aspects of this disease. Changes in the fragility of the erythrocytes are of interest not only from the standpoint of establishing a differential diagnosis between this disease and hemolytic icterus but also because of the important role played by the liver in the maturation of erythrocytes. It is conceivable that the diffuse liver damage in acute infectious hepatitis may be reflected in an altered resistance of erythrocytes to hemolysis by hypotonic saline solutions. Little evidence is available in the current literature on acute infectious hepatitis regarding the effects of this disease on the fragility of erythrocytes. Most reports make no mention of studies of fragility. Havens,¹ however, obtained normal values for erythrocyte fragility in forty-two patients with infectious hepatitis. McCarty² agreed that this value is usually normal. Finks and Blumberg³ carried out sixty-six tests of erythrocyte fragility and concluded that "none of these tests revealed any increased hemolysis as compared with that of the control. In fact a majority of the tests revealed an increased resistance of the red blood cell to hypotonic saline solutions."

In contrast to the majority of these reports which lead one to believe that the changes in erythrocyte fragility are of little uniformity or significance, Cohen,⁴ in a study of twenty-three cases of acute infectious hepatitis, found that "clinical jaundice is accompanied by increased resistance to hypotonic hemolysis" and concluded that "the increased mean resistance is a direct function of the degree of jaundice maintained with and subsiding as does the latter." Our studies have confirmed the finding that the erythrocyte fragility is consistently decreased in acute infectious hepatitis. Further studies were conducted in an attempt to determine the cause of this altered fragility.

MATERIALS AND METHOD

This work was carried out in a United States Army Hospital assigned to a Dutch Hospital Ship operating in the Southwest Pacific. The patients were American soldiers who had developed acute infectious hepatitis in the Philippine Islands and were studied during their evacuation to General Hospitals on New Guinea, a trip which took four or five days. Icteric indices and erythrocyte fragilities were determined on a total of forty-seven patients during three different evacuations, and, as the findings were similar for these three groups of patients, the data will be presented together.

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TABLE I

	PER CENT SODIUM CHLORIDE	
	RANGE	MEAN
Initial hemolysis	0.41 to 0.45	0.429
Complete hemolysis	0.31 to 0.35	0.323
Mean hemolysis	0.360 to 0.395	0.376
Hemolysis span (difference between initial and complete hemolysis)	0.08 to 0.13	0.105

Erythrocyte fragilities were determined by a method outlined by Cohen.⁵ Two-tenths of a cubic centimeter of blood was suspended in 5 c.c. of each of a series of concentrations of saline solution varying from 0.18 to 0.45 per cent. After these suspensions had stood in an icebox for three hours, the initial hemolysis was read as the highest saline concentration to show evidence of hemolysis in the supernatant fluid. The value of complete hemolysis was determined by counting the unhemolyzed cells. The highest saline concentration to contain less than twenty cells per high power field was called the level of complete hemolysis. Using this method, the values shown in Table I were obtained for fifteen normal male controls.

RESULTS

The duration of the disease at the time the first laboratory tests were carried out varied from two to forty-seven days, only nine of the patients being symptomatic for over three weeks. In Fig. 1 is shown the ieteric index in Curve A and the mean erythrocyte fragility in Curve B, plotted against the duration of the disease in days for the forty-seven patients. The ieteric index curve rises and the erythrocyte fragility curve falls during the first week of the disease. Both values reach the peak of abnormality in the second five-day period, then gradually return to normal. The mean erythrocyte fragility does not rise to normal (0.36 per cent) until after the third week of the disease. This corresponds closely to the return to normal of the ieteric index.

Represented in Fig. 2 are changes in ieteric index and mean erythrocyte fragility observed in a hospital ship corpsman who was studied throughout the course of the illness. Fortunately his blood had been used as a control for an erythrocyte fragility test five days before the onset of symptoms. At that time the mean erythrocyte fragility was found to be 0.375 per cent. The results of the laboratory tests on this patient correspond closely to mean curves seen in Fig. 1.

In Fig. 3 erythrocyte fragilities are plotted against their respective ieteric indices for the forty-seven patients studied. Curve A represents the initial hemolysis at different ieteric index levels. This curve falls gradually, dropping below the lower limit of normal at an ieteric index of 19. The level of complete hemolysis, on the other hand, falls much more abruptly, as recorded in Curve B. This relatively more rapid fall in the level of complete hemolysis, as compared to the fall in initial hemolysis, is reflected in a marked increase in fragility span, represented in Curve C.

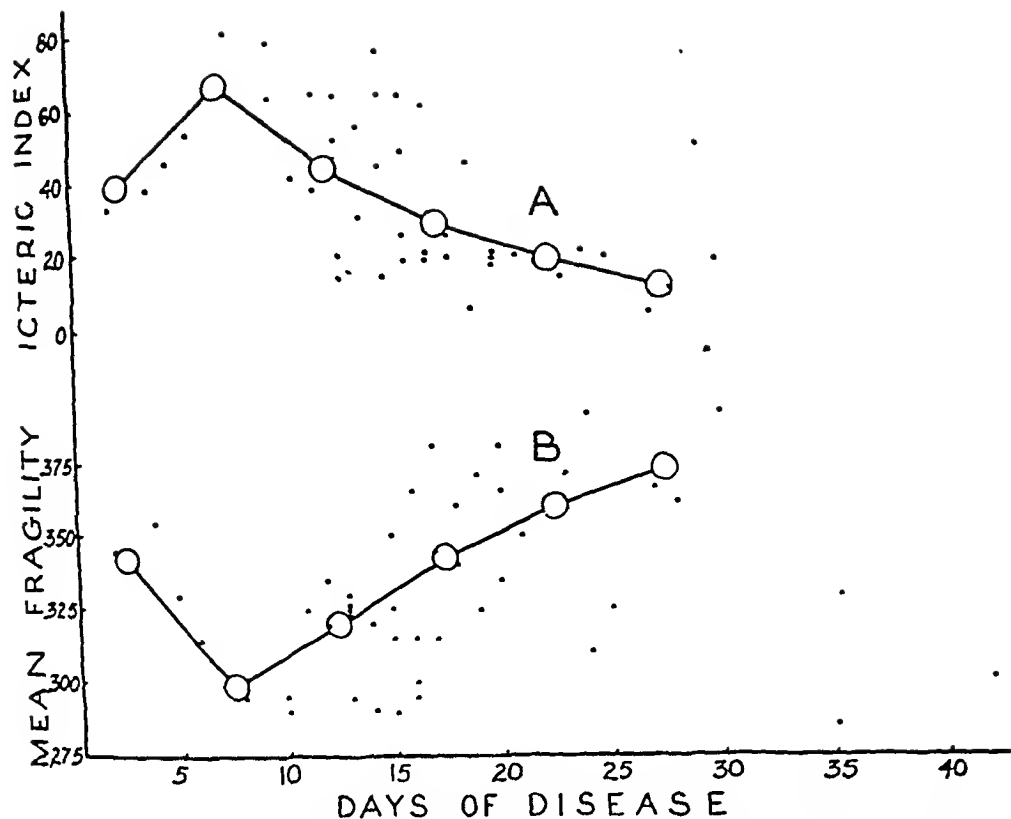


Fig. 1.—Icteric index (Curve A) and mean erythrocyte fragility (Curve B) in forty-seven patients with acute infectious hepatitis, plotted against duration of the disease in days. Curves are constructed on the mean values for each five-day period.

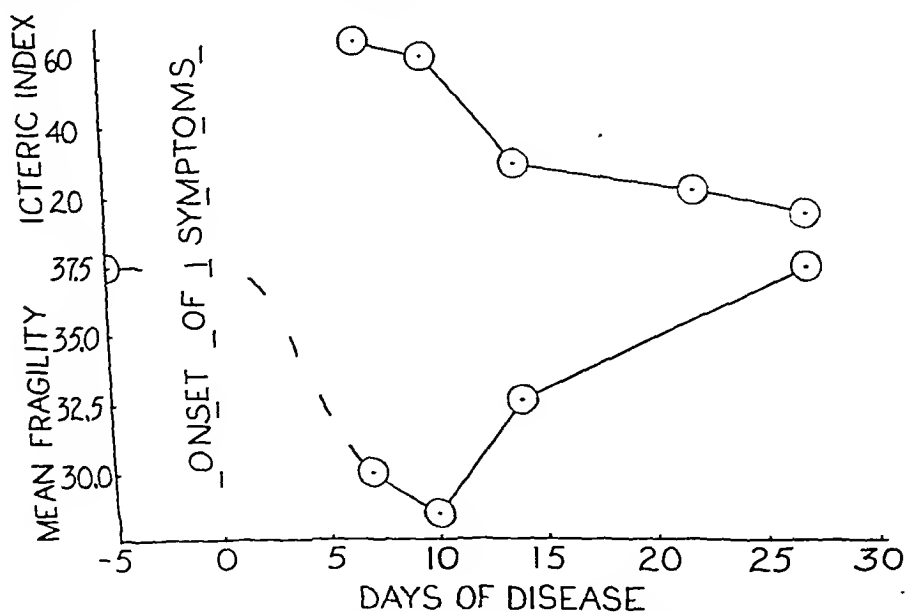


Fig. 2.—Icteric index and mean erythrocyte fragility changes in a hospital corpsman observed throughout the course of acute infectious hepatitis. His blood had been used as a control for an erythrocyte fragility study five days before the onset of symptoms.

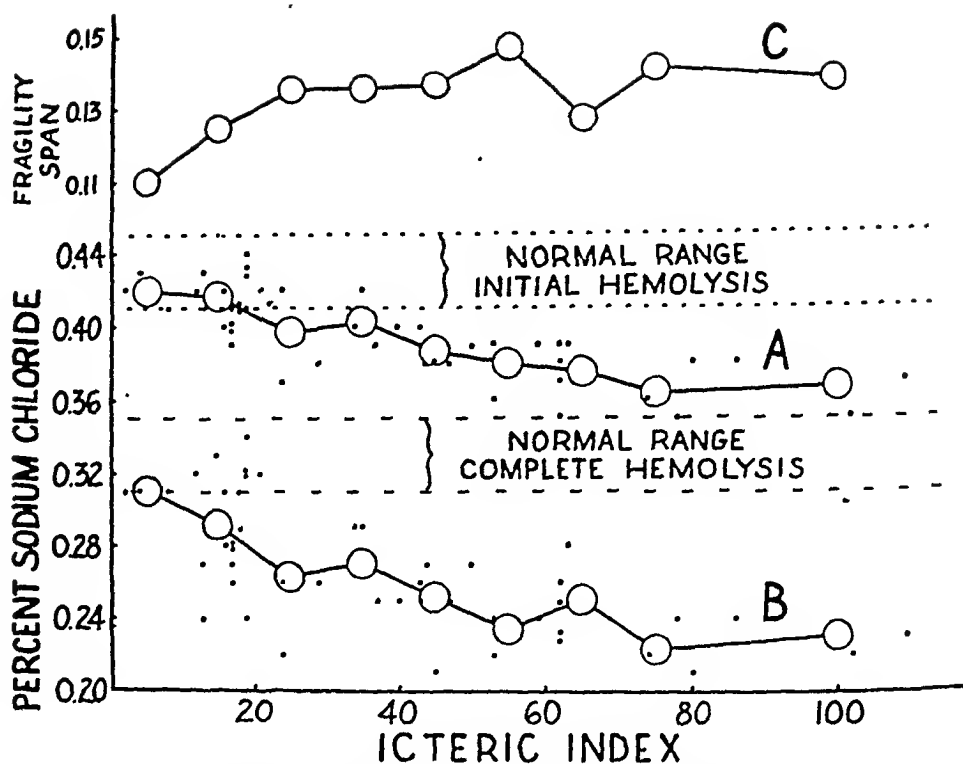


Fig. 3.—Erythrocyte fragility changes plotted against the icteric index for forty-seven patients with acute infectious hepatitis. Curve A is the initial hemolysis, Curve B is the complete hemolysis, and Curve C is the fragility span. Curves are constructed on the mean values for each 10 units of the icteric index.

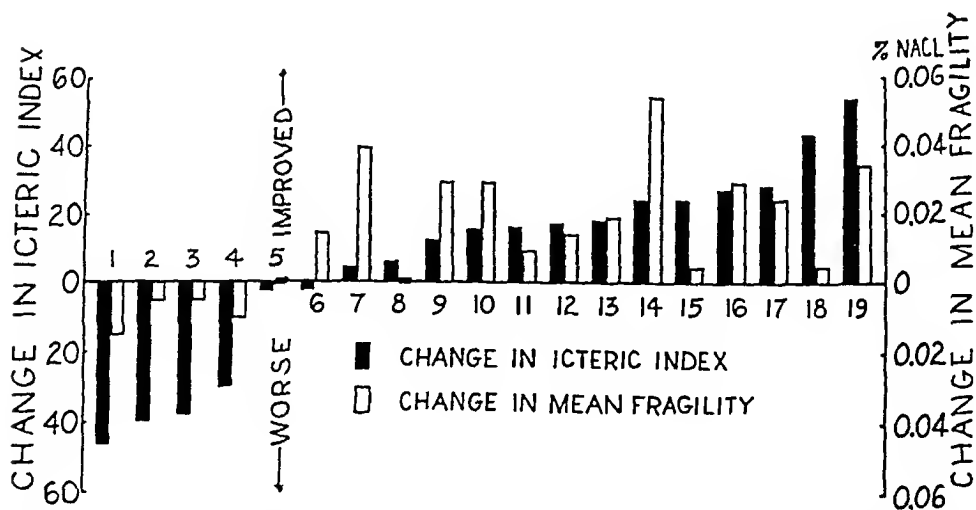


Fig. 4.—Changes in icteric index and mean erythrocyte fragility in nineteen patients with acute infectious hepatitis during a four- or five-day observation period.

DISCUSSION

The fact that erythrocyte fragility is decreased during the icteric phase of acute infectious hepatitis naturally suggests that the bile pigments of the serum may in some way increase the resistance of the cell to hemolysis by hypotonic saline solutions. This possibility has been suggested by Cohen⁴ and Berk.⁶

Several observations concerning the validity of this hypothesis may be made from the data presented here. If the decreased fragility were due to an increase in bile pigment of the serum, changes in these two values in a given patient should be of proportionate magnitude. However, taking several examples from Fig. 3, marked discrepancies are found. For instance, one patient had an icteric index of 13 and complete hemolysis at 0.24 per cent, while another patient with an icteric index of 21 had erythrocytes that were completely hemolyzed within the normal range (0.32 per cent). Another striking example of the disproportionate changes in these two values is seen by comparing the patient with an icteric index of only 24 whose complete hemolysis occurred at 0.22 per cent with the patient who had an icteric index of 63 and complete hemolysis at 0.28 per cent.

Further evidence substantiating this lack of correlation is seen in the progress chart in Fig. 4. Here the magnitude of the changes in erythrocyte fragility is compared with that of the changes in the icteric index during the four- or five-day trip. "Improved" signifies a decreased icteric index and an increased erythrocyte fragility, while "worse" indicates reverse changes. Solid columns represent changes in icteric index, while hollow ones signify changes in erythrocyte fragility. The columns of the nineteen patients thus studied are arranged in the order of increasing improvement in the icteric index. In the first six patients the icteric index actually increased during the trip. The seventh patient showed a marked improvement in erythrocyte fragility and only minimal improvement in icteric index. The reverse situation is equally striking in Patients 15 and 18 where there was marked improvement in icteric indices with only minimal improvement in erythrocyte fragility. Thus it would appear that though both values follow the same general trend the magnitudes of the changes are not related.

From these two observations it may be concluded that the erythrocyte fragility is not a function of the bile pigment concentration of the serum as measured by the icteric index. Nevertheless, in this disease there are other changes in serum chemistry (for example, cholesterol) which may be considered capable of altering the erythrocyte fragility. The laboratory facilities available to us at that time did not permit further chemical studies, but a direct approach to this problem proved enlightening. Normal washed erythrocytes were incubated in homologous serum from patients with acute infectious hepatitis, and conversely, washed cells of decreased fragility from a patient with hepatitis were incubated in normal homologous serum. In Table II it is demonstrated that a three-hour incubation at 38° C. failed to cause a significant change in fragility of either the normal cells or the cells from the patient with

hepatitis. It may be concluded that the decrease in erythrocyte fragility seen in acute infectious hepatitis is not caused by the direct action of any chemical component of icteric serum on normal erythrocytes. The implication that there is some intrinsic defect in these erythrocytes is substantiated by the fact that these low fragility (abnormal) cells from patients with acute infectious hepatitis failed to return to normal when incubated in normal serum. The possibility demonstrated by Haden⁷ that this decreased fragility during jaundice is due to a decrease in the ratio of thickness to diameter of the erythrocytes was not investigated but would seem to supply an explanation for the fragility change.

TABLE 11

	BEFORE INCUBATION		AFTER INCUBATION	
	INITIAL HEMOLYSIS	COMPLETE HEMOLYSIS	INITIAL HEMOLYSIS	COMPLETE HEMOLYSIS
Normal cells placed in serum with icteric index of 122	44	32	44	33
Cells of decreased fragility (from a patient with hepatitis) placed in normal serum	37	23	38	23

Further evidence supporting the hypothesis that the decreased erythrocyte fragility is due to an intrinsic malformation of the cell, or to failure in erythropoiesis, is seen in the increased fragility span in infectious hepatitis (Fig. 3, Curve C). While some cells maintain a normal high degree of fragility, so that the curve of initial hemolysis falls only slightly below normal, other cells are produced which have a strikingly reduced fragility, causing the levels of complete hemolysis to fall precipitously. These disproportionate changes in initial and complete hemolysis cause an increase in fragility span, a manifestation of an increased heterogeneity in the cell population. If decreased erythrocyte fragility were due to the direct action of an extrinsic factor (such as bile pigment or cholesterol) on cells that are otherwise normal, it could be assumed that all cells would undergo an equal decrease in fragility. Initial and complete hemolysis levels would fall the same extent, and the observed increased fragility span would not have been anticipated.

Thus, all the evidence points away from the direct action of some extrinsic factor in the serum and toward a faulty erythropoiesis as being responsible for the decreased erythrocyte fragility in acute infectious hepatitis. It is conceivable that diffuse liver damage may cause this fault in erythropoiesis. If this interpretation is correct, changes in liver function are manifested by altered erythrocyte fragility; the results of this simple test, which have been found to be consistently abnormal in acute infectious hepatitis, may serve as another index of the degree of liver damage.

CONCLUSIONS

1. The fragility of erythrocytes in acute infectious hepatitis is consistently decreased.
2. Evidence is presented which indicates that this decreased fragility is not a function of the degree of jaundice.

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THE TREATMENT OF MACROCYTIC ANEMIA WITH LACTOBACILLUS CASEI FACTOR (PTEROYLGLUTAMIC ACID)

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SINCE *Lactobacillus casei* factor (folie acid) was synthesized in 1945 a number of reports have appeared indicating the usefulness of this vitamin in the treatment of human macrocytic anemia. Darby, Jones, and Johnson,¹ Spies and co-workers,² Moore and associates,³ Goldsmith,⁴ and Doan, Wilson, and Wright⁵ have found *L. casei* factor to be effective in the treatment of sprue, pernicious anemia, nutritional macrocytic anemia, and the macrocytic anemia of pregnancy. The structure of *L. casei* factor has recently been elucidated and the name pteroylglutamic acid suggested for this compound.⁶

This report deals with the use of pteroylglutamic acid in fifteen persons with anemia, five of whom have been studied for more than six months. Two patients with pernicious anemia, four with nutritional macrocytic anemia, and one with macrocytic anemia in which the bone marrow was depressed showed marked clinical and hematologic improvement when *L. casei* factor was given as the sole therapeutic agent. Two patients with sprue and one with a celiac syndrome improved in certain respects, but the blood picture was altered in only one instance. *L. casei* factor was without beneficial effect in two patients with aplastic anemia and in one patient each with macrocytic anemia associated with regional ileitis and myxedema and macrocytic anemia of unknown origin.

METHOD OF STUDY

Patients with anemia were hospitalized for the first month or more of study and in most instances were placed on diets low in protein and containing no meat. Complete blood counts were obtained daily at first and later at increasing intervals. Blood was obtained by venipuncture without stasis, and approximately 5 c.c. were placed in tubes containing ammonium and potassium oxalate. United States Bureau of Standards equipment was used for all blood counts. Hemoglobin was determined by the Newcomer method, and the volume of packed erythrocytes was measured with the Wintrobe hematocrit tube. Reticulocyte stains were made with the wet film technique. Other laboratory determinations in this study included urinalyses, serologic tests for syphilis, gastric analyses after histamine stimulation, stool examinations, glucose tolerance tests, roentgenologic examinations of the chest and gastrointestinal tract, bone marrow biopsies, and renal function and vitamin excretion tests.

L. casei factor was administered either orally or parenterally in amounts which varied from 5 to 120 mg. daily. A few patients who failed to respond satisfactorily to *L. casei* factor were given pyridoxine, calcium pantothenate, iron, or liver extract as additional therapy.

The clinical condition of each patient was checked at frequent intervals, and laboratory procedures were repeated as often as seemed advisable. The period of time during which patients have been followed has ranged from four weeks to more than six months. One patient has been studied at intervals for over two years.

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RESULTS

Some of the pertinent laboratory and clinical findings in the fifteen patients with anemia who were treated with *L. casei* factor are given in Table I. The diagnoses which were made as to type of anemia are also indicated. The criteria which were used in diagnosing pernicious anemia were those which are generally accepted, including absence of free hydrochloric acid from the gastric contents after histamine stimulation, a macrocytic anemia without demonstrable primary cause, and a hyperplastic bone marrow containing large numbers of megaloblasts. Good nutrition, elevation of serum bilirubin, and mild neurologic changes were usually present.

The diagnosis of sprue was made when there was steatorrhea, loss of weight, a macrocytic anemia with bone marrow findings similar to pernicious anemia, a flat glucose tolerance test, and a deficiency pattern on x-ray examination of the small intestine. Pigmentation of the skin and gastric achlorhydria were found in one patient.

The term nutritional macrocytic anemia was applied to patients who had been receiving inadequate diets and in whom macrocytic anemia occurred in association with symptoms and signs of deficiency of vitamins of the B complex. Diarrhea, glossitis, and loss of weight were constant findings. Free hydrochloric acid was often present in the gastric juice after histamine stimulation. Fat in the stools was not increased. The bone marrow varied, being either hypercellular or depressed, and normoblasts or pronormoblasts were the predominant cell in some instances, rather than megaloblasts. Macrocytosis was usually less prominent than in pernicious anemia of similar severity. Anemia of nutritional origin may respond to yeast or to a good diet without special medication.

The seven patients with macrocytic anemia whose condition improved with the administration of pteroyl glutamic acid responded to treatment in a characteristic manner. Reticuloocytes in the blood increased at the end of two to six days reaching a peak after five to eleven days. The rise was not as great as would have been anticipated had liver extract been administered, varying from 5 to 12 per cent. Similar findings were recently reported by Heinle, Welch, and Nelson.⁷ The increase in reticuloocytes often persisted for long periods of time especially if large amounts of *L. casei* factor were given daily. The total erythrocyte count and the percentage of hemoglobin began to rise within a week to ten days, the maximum being attained in twenty-four to forty-three days when the amount of *L. casei* factor given was 15 mg. daily. The initial leucocyte count was low in three instances and rose to normal in seven to fourteen days after therapy had been started. The number of platelets likewise increased. Four patients have been maintained in good clinical condition and with a relatively normal blood picture for over six months.

In four instances (Patients 3 to 5 and 7) the bone marrow was examined both before and after therapy with *L. casei* factor. Regardless of whether the initial findings were those of depression or stimulation there was a return to normal after six to eight weeks of treatment.

Findings in Pernicious Anemia.—The diagnosis of pernicious anemia was made in two patients. The first was a white woman, 55 years of age, who gave

TABLE I. CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH ANEMIA PRIOR TO THERAPY

PATIENT	RACE	SEX	AGE	TYPE OF ANEMIA	BLOOD PICTURE					BONE MARROW	FREE HCL	DIET	OTHER FINDINGS
					R.B.C. (MILL- IONS)	Hb. (GM.)	HCT.* (%)	MCV†	YCHCT†				
1	W	F	55	Pernicious	1.7	6.2	20.5	121	30	Hypereellular megablasts	0	Good	Numbness, fingers
2	W	M	57	Pernicious	3.7	11.0	37.0	100	30		0	Fair	Numbness; vibra- tory sense and reflexes ↓ legs
3	C	M	40	Undetermined (pernicious?)	1.7	4.9	14.0	82	35	Depressed, few megablasts	0	Fair	Numbness, finger-
4	W	F	68	Nutritional macrocytic	2.3‡	7.0	22.0	96	32	Hypereellular pro- normoblasts	0	Very poor	Emaciation, diar- rhea, stupor
5	W	F	67	Nutritional macrocytic	1.8	6.0	20.0	111	30	Cellular, erythroid series depressed, normoblasts,	35	Poor	Weight loss, edema, diarrhea, mental con-
6	W	F	64	Nutritional macrocytic	2.3	8.4	20.0	126	35	Cellular, no megablasts	0	Very poor	Edema, poly- neuritis, emacia- tion, diarrhea, irritability
7	W	F	69	Nutritional macrocytic	1.7	5.0	15.0	88	33	Depressed, many megablasts	26	Poor	Numbness
8	W	F	55	Sprue	3.5	11.5	39.0	111	29			Fair	Statorrhea, weight loss
9	W	F	54	Sprue	3.6	11.3	38.0	106	30	Hypereellular pro- normoblasts, normoblasts	0	Fair	Diarrhea, stool fat increased
10	W	M	4	Celiac syndrome	3.9	11.0	38.0	97	29			Poor	Emaciation, statorrhea
11	C	M	63	Undetermined	2.1	6.8	26.0	124	27	Hypereellular pro- normoblasts, normoblasts	0	Very poor	Emaciation Combined sclerosis
12	C	M	51	Aplastic	0.8	2.8	10.0	125	28	Depressed lymphocytes	0	Fair	Glossitis minimal
13	W	M	8	Aplastic	2.2‡	5.6	18.0	82	31	Predominant Depressed, few normoblasts	20	Fair	
14	W	F	50	Myxedema	3.2	10.2	32.0	100	32			Good	
15	W	M	30	Regional ileitis	3.1	10.2	35.0	103	29			Good	Diarrhea

All patients had glossitis except Patients 11, 14, and 15.
*Hct., Hematocrit-volume packed erythrocytes

a history of dyspnea, palpitation, and dizziness for six months prior to entering the hospital in November, 1945. She had diarrhea and vomiting at intervals and had lost weight, although she was still obese. Weakness, numbness of the fingers, and a yellowish discoloration of the skin had been present for one month. The patient stated that her sister was being treated for anemia with liver extract. Physical examination showed moderate obesity, a pale lemon-yellow skin, a smooth red tongue with atrophic papillae, slight cardiac enlargement, and a systolic murmur audible in all cardiac valve areas. Neurologic examination was

TABLE II. HEMATOLOGIC RESPONSE OF PATIENTS WITH PERNICIOUS ANEMIA TO *L. Casei* FACTOR

PATIENT	DAYS AFTER BEGINNING THERAPY	R.B.C. (MILLIONS)	Hb. (GM.)	HCT.* (% R.B.C.)	AMOUNT <i>L. Casei</i> FACTOR PER DAY	DIET
1	0	1.7	6.2	20.5	15 mg. I.M.,† 20 days	Low protein, meat free
	31‡	3.5	10.0	35.0	None	Low protein, meat free
	47	3.1	9.5	36.0	15 mg. Orally	Low protein, meat free
	90‡	4.0	12.0	40.0	15 mg. Orally	Regular
	160	4.0	11.2	40.0	15 mg. Orally§	Regular
	188	4.3	11.9	41.0	15 mg. Orally	Regular
2	0	3.5	10.5	36.0	120 mg. Orally, 6 days	Low protein
	9	4.0	11.9	39.0	30 mg. Orally	Low protein
	24	4.2	13.5	44.0	30 mg. Orally	Regular
	56‡	5.8	18.9	48.0	30 mg. Orally	Regular
	84	6.0	16.6	5.5	5 mg. Orally	Regular
3	0	1.7	4.9	14.0	15 mg. I.M.,† 19 days	Regular
	43‡	4.0	12.0	39.5	None	
	57	3.5	9.4	39.0	None	
	106	4.5	11.5	41.0	None	
	169	3.0	9.6	31.0	None	
	190	2.5	7.8	29.0	None	

*Hct., Hematocrit.

†I.M., Intramuscularly.

‡Maximum rise with given dose of *L. casei* factor.

§Ferrous sulfate added, 1 Gm. daily.

||Left hospital.

normal. The icterus index was 20, while other laboratory findings, except for the anemia, were within normal limits. This patient was placed on a low protein, meat-free diet and was given 15 mg. of *L. casei* factor daily, intramuscularly, for twenty days. The hematologic response is shown in Fig. 1. After forty-seven days it was apparent that no further improvement would occur, and she was given *L. casei* factor orally, 5 mg. three times daily. This medication has been continued for 188 days (Table II). At the end of three months a regular diet was instituted. This had no effect on the blood count which remained slightly below normal with macrocytosis persisting. Iron was added to the therapeutic regimen, and after four weeks improvement of questionable significance occurred. During the seven months in which this patient has been observed she has gained weight and strength, the lingual papillae have regenerated, and numbness of the hands has disappeared. She is without complaints and is working daily in a newspaper office.

The second patient with pernicious anemia was a 57-year-old white man who was treated with liver extract from April, 1944, to December, 1945, at which time he discontinued therapy. He returned to the hospital in April, 1946, complaining of weakness, numbness of the hands and feet, and soreness of the tongue. In addition to a mild macrocytic anemia the positive findings on examination were a pale, smooth tongue, absence of vibratory sensation and

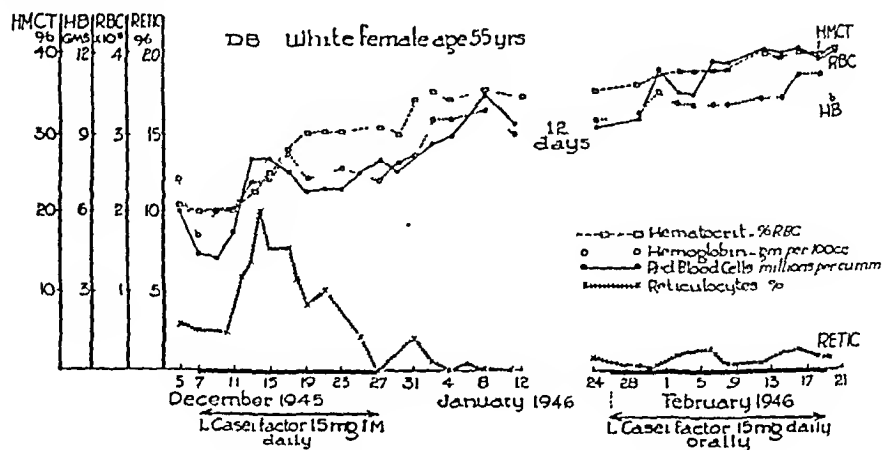


Fig. 1—Pernicious anemia treated with *L. casei* factor

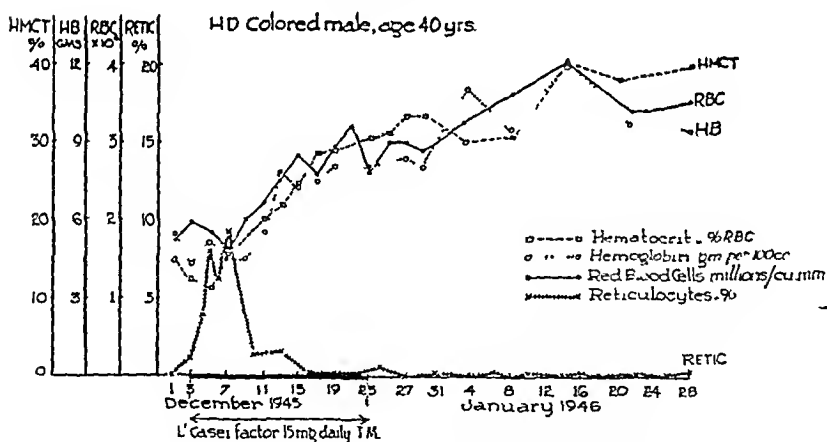


Fig. 2—Hypoplastic anemia treated with *L. casei* factor

diminished reflexes in both lower extremities, achlorhydria following histamine administration and atrophic gastritis involving the pyloric antrum, and pars media of the stomach on gastroscopic examination. The initial blood picture and the response to therapy with *L. casei* factor is shown in Table II. In this patient a very marked increase in both erythrocytes and hemoglobin has occurred. This is the only instance in which high normal levels have been attained. Whether or not the massive dose of *L. casei* factor which was used is responsible for this finding will require further study. The patient feels well, numbness and

weakness have disappeared, reflexes in both legs are normal, and vibratory sensation has returned in the left lower extremity.

The third patient included in this group was at first thought to have aplastic anemia. However, the subsequent course suggests that he, too, has pernicious anemia. He is a colored man, 40 years of age, who entered the hospital in November, 1945, complaining for the past eight weeks of weakness, dyspnea, and substernal distress on exertion. In 1943 he was treated for amebiasis. At that time a severe anemia was discovered but therapy was not instituted. Physical examination in 1945 showed a well-nourished Negro with pallor of the skin and mucous membranes. The tongue was atrophic. There was a faint systolic murmur audible in all cardiac valve areas and the liver was palpable. Several gastric analyses showed achlorhydria. Other laboratory findings including x-ray examination of the chest and gastrointestinal tract were normal, except for anemia which was normocytic and normochromic in type. The bone marrow was markedly depressed; lymphocytes predominated but a few megaloblasts were seen. It was suggested that these findings might represent pernicious anemia in an aplastic phase. *L. casei* factor was administered intramuscularly in amounts of 15 mg. daily for nineteen days. The response to therapy is illustrated in Fig. 2. No further treatment has been given, and the blood count has decreased. Although he feels well at the present time, the patient has a macrocytic anemia of moderate severity (Table II).

Findings in Nutritional Macrocytic Anemia.—Four patients with nutritional macrocytic anemia were treated with *L. casei* factor (Patients 4 to 7). All were white women over 60 years of age who had been eating very limited diets, inadequate in calories and in vitamins of the B complex. Each patient complained of anorexia, soreness of the tongue, diarrhea, and loss of weight. Three patients had free hydrochloric acid in the stomach. Bone marrow findings were variable (Table I). Vitamin excretion tests in each of the four patients showed a subnormal output of thiamine, niacin, and riboflavin.

Patient 4 was extremely weak and emaciated when admitted to the hospital in October, 1945. She had been vomiting for two weeks, was dehydrated and semistuporous, and appeared much older than her actual age of 68. She was incontinent of both urine and feces. The skin was dry and pale. The tongue was red, smooth, and atrophic, and only three carious teeth remained in the mouth. The heart was slightly enlarged to the left, and the electrocardiogram showed low QRS complexes, a prolonged QT interval, and inverted T waves in Lead I. The erythrocyte count on admission was 700,000 per cubic millimeter with an hematocrit reading of 8.5 per cent. Serum proteins were reduced to 5.5 Gm. per 100 c.c. of blood. Other laboratory findings were not remarkable. The patient received three transfusions, a high caloric diet, thiamine, niacin, and riboflavin. She became mentally clear, gained strength, and sphincter control returned. The tongue was unimproved. After a month, this therapy was discontinued and she was placed on a low protein diet. *L. casei* factor was administered in amounts of 15 mg. daily intramuscularly for twenty days. The anemia improved as shown in Table III. The papillae of the tongue regenerated

rapidly. Several months later *L. casei* factor was given orally, 10 mg. three times daily. The blood count at the end of two months of treatment with this large amount of *L. casei* factor is essentially normal. The patient feels well, has gained 35 pounds, and all symptoms of anemia have disappeared.

Patient 5 had noted weakness and swelling of the ankles for nine months before entering the hospital. At the time of admission she was confused, semi-stuporous, and the rectal temperature was 102° Fahrenheit. Blood pressure was 180/60, pulse rate 110. The mouth was edentulous and the tongue purplish red and atrophic. There was slight cardiac enlargement, a blowing systolic murmur in all of the valve areas, occasional premature contractions, râles at the base of the left lung, ascites, and edema of the legs. The calf muscles were extremely tender. Venous pressure and circulation time were slightly elevated. A glucose tolerance test showed a flat curve; serum proteins were normal.

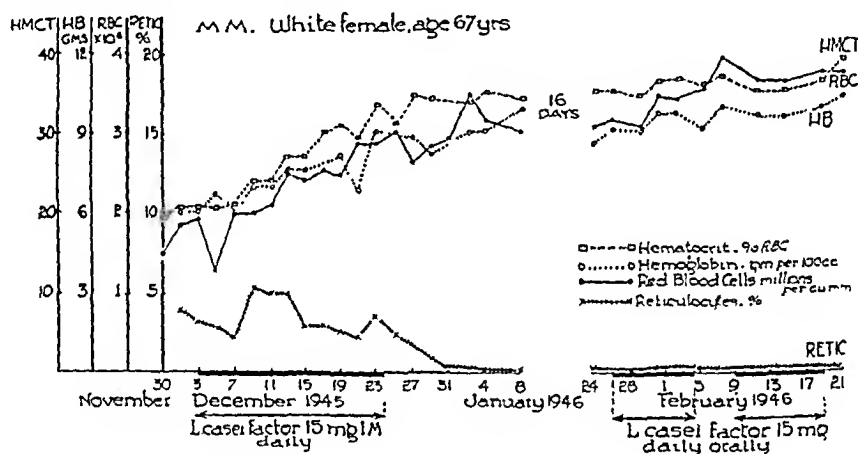


Fig. 3.—Nutritional macrocytic anemia treated with *L. casei* factor.

Liver function as measured by the hippuric acid and cephalin flocculation tests was normal. The urine contained 3 per cent moist albumen. The patient was digitalized and treatment with *L. casei* factor was instituted. The hematologic response to 15 mg. given parenterally daily is shown in Fig. 3. From a clinical standpoint there was rapid improvement. The patient developed a ravenous appetite, gained in weight and strength, and papillae of the tongue regenerated. The edema gradually disappeared and cardiac compensation was restored. Subsequent therapy and the changes which occurred in the blood picture are shown in Table III. *L. casei* factor was effective with either parenteral or oral administration, but the blood count remained below normal. Increasing the amount of *L. casei* factor to 30 mg. a day has not resulted in further improvement.

Patient 6 was first seen in May, 1945, at which time she complained of weakness and burning of the tongue of five months' duration and a loss in weight of 55 pounds. For three months she had noted paresthesias of the toes and for one month diarrhea, three or four watery stools daily, and edema of the

ankles. Examination showed undernutrition, glossitis, a macrocytic anemia of mild degree, and tenderness of the calf muscles. A diagnosis of deficiency of the vitamin B complex was made, and treatment with thiamine, niacin, riboflavin, and brewers' yeast was instituted. After one month the patient was discharged from the hospital much improved.

The patient returned in April, 1946, at which time she was so weak she could hardly stand. She had noted a sore tongue, diarrhea, dyspnea on exertion, edema of the ankles, and painful feet. She was irritable and did not cooperate well. Her diet for five months had consisted largely of rice, grits, and gravy. She weighed 78 pounds. The tongue was red and no papillae were visible. Cheilosis with angular maceration was present. There was definite evidence of peripheral neuritis involving both lower extremities which were also edematous. The heart and lungs were normal. Blood pressure was 95/60.

TABLE III. HEMATOLOGIC FINDINGS IN PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA TREATED WITH *L. Casei* FACTOR

PATIENT	DAYS AFTER BEGINNING THERAPY	R.B.C. (MILLIONS)	Hb. (GM.)	HCT.* (% R.B.C.)	AMOUNT <i>L. Casei</i> FACTOR PER DAY	DIET
4	0	2.3	7.0	22.0	15 mg. I.M.,† 20 days	Low protein, meat free High vitamin, high caloric
	24‡	4.5	11.0	39.0	None	
	103	3.3	10.0	36.0	30 mg. Orally	
	126	3.7	11.6	42.0	30 mg. Orally§	
	179	4.4	14.9	46.0	5 mg. Orally	
	200	4.6	13.0	46.0	5 mg. Orally	
5	0	1.8	6.0	20.0	15 mg. I.M.,† 20 days	Low protein, meat free Regular Regular
	30‡	3.4	9.0	34.0	None	
	54	3.2	9.0	34.5	15 mg. Orally, 19 days	
	93	4.0	10.7	38.0	15 mg. Orally§	
	154	3.9	10.3	38.0	30 mg. Orally	
	189	4.2	10.4	37.0	30 mg. Orally	
6	0	2.3	8.4	29.0	40 mg. I.M.,† 3 times weekly	High protein
	35‡	3.6	10.7	38.0	40 mg. I.M.,† 3 times weekly	
	60	3.7	9.9	37.0	100 mg. Orally, 7 days	High protein
	84	4.0	9.9	37.0	None	

*Hct., Hematocrit.

†I.M., Intramuscularly.

‡Maximum rise with given dose of *L. casei* factor.

§Left hospital.

Roentgenologic examination of the gastrointestinal tract was without abnormal findings. There was achlorhydria after histamine administration and serum proteins were reduced (5.46 Gm. per cent). Liver function tests were normal. There was a moderately severe macrocytic anemia. *L. casei* factor was administered in amounts of 40 mg. intramuscularly three times weekly, and a high protein diet was instituted. The anemia improved (Table III) but a completely normal blood picture was not attained. After receiving 100 mg. of *L. casei* factor orally every day for one week there was only a slight additional rise in the blood count. In spite of the persistence of mild anemia the patient

has improved remarkably. Edema has disappeared, papillae of the tongue have regenerated, and cheilosis has healed. Thiamine was administered in amounts of 10 mg. three times daily after the first two weeks in the hospital, and there has been complete recovery from the peripheral neuritis. The patient has gained 28 pounds.

Patient 7 has been followed for more than two years. When first seen in October, 1943, she complained of anorexia, diarrhea, and vomiting for six months. She had lost 70 pounds. She was undernourished, pale, edentulous, and had an atrophic tongue. The heart was not enlarged. There was a soft blowing apical systolic murmur and occasional premature contractions. Peripheral blood vessels were extremely sclerotic. She had a macrocytic anemia, leucopenia, and thrombopenia. There was free acid in the stomach. Extensive investigation showed no evidence of malignancy, infection, or disease of the liver. Since 1944 this patient has been treated at intervals with various anti-anemic substances. In April, 1944, 5 mg. of folic acid concentrate and 100 of calcium pantothenate were given daily for one week. Reticuloeytes increased to 12 per cent on the ninth day; this was followed by an increase in erythrocytes and hemoglobin (Table IV). Folic acid concentrate was given

TABLE IV. HEMATOLOGIC FINDINGS IN PATIENT WITH NUTRITIONAL MACROCYTIC ANEMIA TREATED WITH "FOLIC ACID" AT INTERVALS FOR TWENTY-SIX MONTHS

DATE	BLOOD PICTURE			MEDICATION† (AMOUNT GIVEN DAILY)
	R.B.C. (MILLIONS)	HB. (GM.)	HCT.* (% PACKED R.B.C.)	
4/23/44	1.7	5.0	15.0	Folic acid concentrate,‡ 5 mg., and calcium pantothenate, 100 mg.; 4/23/44 to 4/30/44
5/ 8/44	2.5	7.5	26.0	
5/29/44	2.7	7.5	28.0	
10/ 7/44	3.3	11.5	34.5	Folic acid concentrate,‡ 5 mg.; 10/7/44 to 10/14/44
10/28/44	4.1	13.0	39.0	
6/ 8/45	2.1	9.1	28.5	Crystalline folic acid, 5 mg., and calcium pantothenate, 10 mg., 6/8/45 to 6/15/45
7/12/45	3.0	11.5	36.0	
10/19/45	2.3	9.5	24.5	<i>L. casci</i> factor, 5 mg.; 10/19/45 to 10/28/45
11/ 9/45	2.5	10.5	31.0	<i>L. casci</i> factor, 5 mg., and calcium pantothenate, 10 mg.; 11/9/45 to 11/18/45
11/23/45	2.9	11.5	34.5	<i>L. casci</i> factor, 15 mg., intramuscularly; 11/23/45 to 11/29/45
12/ 1/45	4.2	10.5	35.0	<i>L. casci</i> factor, 15 mg.; 1/20/46 to 2/10/46
12/17/45	3.0	11.5	37.5	
1/20/46	3.1	10.0	38.0	
2/ 9/46	4.1	10.2	37.5	<i>L. casci</i> factor, 30 mg.
2/20/46	3.4	10.5	37.5	
3/18/46	3.9	11.0	37.5	
4/22/46	4.0	11.0	38.5	<i>L. casci</i> factor, 30 mg.
5/27/46	4.1	12.0	39.0	<i>L. casci</i> factor, 30 mg., and pyridoxine, 30 mg.
6/24/46	4.2	11.3	39.0	<i>L. casci</i> factor, 30 mg., and pyridoxine, 30 mg.

*Hct., Hematocrit.

†Given orally except 11/23/45 to 11/29/45.

‡"Fermentation" folic acid.

again in October, 1944. A slight improvement in the blood picture occurred. Crystalline folic acid was followed by a similar hematologic response in June, 1945. Since synthetic *L. casei* factor has been available it has been administered in varying dosage on several occasions, either alone or in combination with calcium pantothenate or pyridoxine. In most instances there has been hematologic improvement with *L. casei* factor, although large doses have not brought about an increase in erythrocytes above 4 to 4.2 million or of hemoglobin above 11 to 12 grams. It is of course possible that this is a normal blood picture for this patient. In these studies there is no definite evidence that calcium pantothenate or pyridoxine influenced blood regeneration.

This patient has been treated also with liver extract, yeast, iron, and individual vitamins of the B complex. The hematologic changes over the period of one and one-half years with various medications are shown in Fig. 4. Oral liver extract given after the administration of folic acid concentrate was without effect on the blood picture, while concentrated liver extract given parenterally brought about a marked rise in erythrocytes and hemoglobin. On one occasion brewers' yeast, three ounces daily, was given for five weeks without effect. There was no improvement with 1 Gm. of ferrous sulfate daily for two months or with 15 mg. thiamine, 150 mg. niacin, and 5 mg. riboflavin daily for one month.

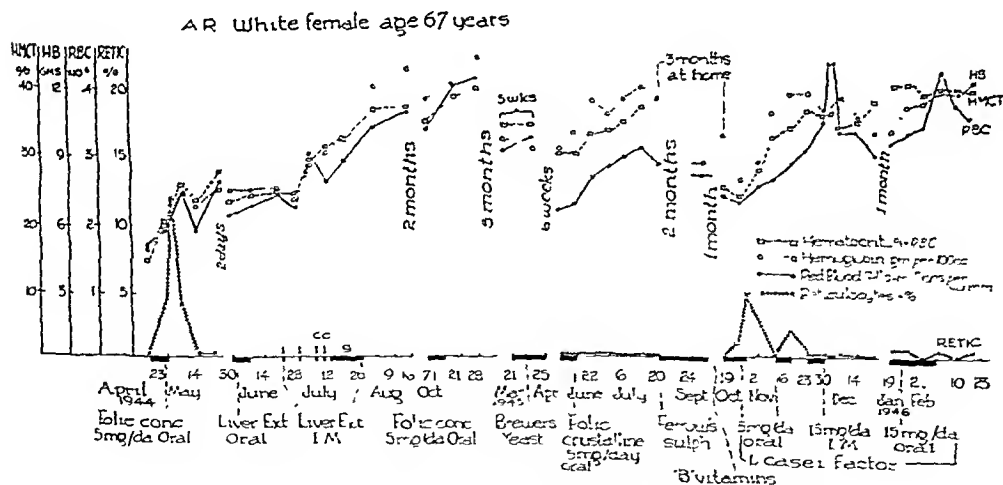


Fig. 4.—Hematologic response to various therapeutic agents in a patient with nutritional macrocytic anemia. (April 24, 1944, through Feb. 23, 1946.)

Findings in Sprue.—Two patients with sprue have been treated with *L. casei* factor. Patient S (Table I) has been known to have sprue for fourteen years. In 1932 at the time the original diagnosis was made, her weight had decreased from 150 to 55 pounds. She had steatorrhea, abdominal distention, glossitis, ulcers of the mouth, profound anemia of macrocytic type, and weakness so marked that she could scarcely move in bed. Since that time she has been eating a diet high in protein and bananas and has received liver extract. Whenever liver has been omitted the blood count has fallen and symptoms of anemia have

reappeared. The steatorrhea has never been completely controlled. Even when the blood count was high (erythrocytes 4.5 to 5 million) she has had occasional bulky, frothy stools containing large amounts of fat and fatty acid. In April, 1945, liver extract was discontinued, the diet remaining constant. By January, 1946, she had lost 7 pounds, stools had become increasingly fatty, the tongue had become sore, and the blood count had fallen slightly (Table I). Roentgenologic examination of the small intestine showed a typical deficiency pattern. She felt weak and was unwilling to remain without therapy for a longer period of time. *L. casei* factor, 50 mg., was given daily by mouth, and meat was withdrawn from the diet, which did, however, include bananas and milk. Within three days she felt stronger. The bowels improved markedly and she had only one large stool daily which contained less fat. Soreness of the tongue was relieved and papillae reappeared on the sides and tip. The patient regained her lost weight. The blood count showed no change at the end of three weeks. *L. casei* factor was given intramuscularly, at first 20 mg., then 40 mg. three times a week for a total of five weeks. The blood count remained essentially the same. However, the percentage of reticulocytes which had been 0.2 to 2 before therapy ranged from 1 to 4 per cent when *L. casei* factor was administered. Meat was added to the diet without any effect on the anemia. The addition of pyridoxine to the regimen was also without benefit. At the end of three and one-half months, therapy with *L. casei* factor was discontinued and injections of concentrated liver extract (30 units) were given three times a week for four weeks. The percentage of hemoglobin and volume of packed red cells remained the same, but the erythrocyte count increased from 3.6 to 4 million per cubic millimeter. Reticulocytes varied from 4 to 6 per cent. This slight change suggests a decrease in macrocytosis.

The second patient with sprue (Patient 9) was first seen in August, 1945. She had a history of diarrhea of fatty type for two years, a loss of 15 pounds in weight in the previous four months, and burning of the tongue. A glucose tolerance test showed a flat curve, and there was histamine refractory achlorhydria. The stools contained large amounts of fat, and examination of the blood showed a macrocytic anemia. Marked improvement occurred with a diet high in protein, low in fat and carbohydrate, and injections of crude liver extract. Treatment was stopped in October, 1945, and in April, 1946, the patient had again developed fatty diarrhea, a sore tongue, and had lost weight. The blood count at that time is given in Table I. *L. casei* factor, 30 mg., was given orally daily for three weeks, at which time erythrocytes were 3.8 million per cubic millimeter, hemoglobin 11.6 Gm., and the volume of packed erythrocytes 40.5 per cent. Calcium pantothenate, 30 mg. daily, was prescribed in addition to *L. casei* factor for another three weeks, at which time erythrocytes were 3.9 million per cubic millimeter, hemoglobin 11.3 Gm., and the volume of packed red cells 40 per cent. Although the anemia was not significantly influenced by therapy, the diarrhea was controlled, soreness of the tongue disappeared, and the patient gained 6 pounds. Therapy was then changed to liver extract, 15 units being given three times the first week and 30 units three times a week for the subsequent two

weeks. Reticulocytes increased from 3 to 6 per cent, while erythrocytes and hemoglobin rose to 45 million per cubic millimeter and 12 Gm. per cent, respectively. The volume of packed red cells increased to 41 per cent.

One patient with a celiac syndrome was treated with *L. casei* factor (Patient 10). He was a 4-year-old white boy who was underdeveloped and emaciated and who had had steatorrhea since the age of two or three weeks. A diagnosis of pancreatic fibrosis was considered but not proved. Anemia was normocytic and normochromic and failed to improve with iron. When *L. casei* factor was administered the stools became less frequent and more formed. There was a slight gain in weight. Hematologic studies showed the following after thirteen daily injections of 20 mg. of *L. casei* factor; erythrocytes had increased from 3.9 to 4.5 million per cubic millimeter, hemoglobin from 10.9 to 12.5 Gm. per cent, and volume of packed red cells from 38 to 40.5 per cent. This change, while small, seemed definite.

Other Types of Anemia.—Two patients with aplastic anemia (Patients 12 and 13, Table I) received *L. casei* factor without benefit. Patient 13 was an 8-year-old white boy; Patient 12 was a colored man, 51 years of age. In neither instance could any cause of the anemia be discovered in spite of extensive investigation. In one instance (Patient 12) the anemia was macrocytic. This patient was given 5 mg. and then 30 mg. of *L. casei* factor daily. At the end of one month there had been no improvement and it had been necessary to give several blood transfusions. Patient 13 received 20 mg. of *L. casei* factor parenterally daily for ten days, then 40 mg. daily for a similar period. There was no change in the blood picture, and seven blood transfusions were needed in a period of fifty days.

Patient 11 (Table I) had a macrocytic anemia, the cause of which could not be determined. On admission to the hospital, the tentative diagnosis was pernicious anemia with degeneration of the posterior and lateral columns of the spinal cord. The patient was an emaciated colored man who was extremely weak. There was loss of vibratory and position sensibility and absence of the deep reflexes in both lower extremities. The Babinski reaction was positive on the left, and there was loss of sphincter control. There was a macrocytic anemia which was hypochromic suggesting that iron deficiency was complicating the picture. The patient had no glossitis, and the bone marrow while hypercellular did not contain megaloblasts. Physical examination and numerous laboratory studies including spinal puncture and x-rays of the chest and gastrointestinal tract failed to show any evidence of neoplasm or infectious disease. *L. casei* factor was given in amounts of 15 mg. daily for two weeks without any effect. The patient developed a urinary tract infection and bed sores and died after one month in the hospital. An autopsy could not be obtained.

Patient 14 who had myxedema and a mild macrocytic anemia was given 30 mg. of *L. casei* factor daily for fifteen days without benefit, which finding was not unexpected. She also failed to improve with liver extract.

Patient 15 had regional ileitis with a mild anemia which had improved somewhat with therapy with liver extract. It was felt that *L. casei* factor might

benefit the mild macrocytic anemia which persisted; therefore, liver injections were discontinued and *L. casei* factor was given in 20 mg. doses intramuscularly at intervals for five weeks, a total of 500 mg. being administered. There was no change in the blood picture.

DISCUSSION

The preceding case reports indicate that *L. casei* factor (pteroylglutamic acid) is a potent hemapoietic substance in pernicious anemia and in anemia of nutritional origin in which macrocytosis is a feature. Clinical improvement paralleled hematologic response in all instances. A gain in weight and strength and relief of glossitis with regeneration of lingual papillae occurred. These findings are corroborative of those reported by other investigators. In addition, one patient with pernicious anemia had neurologic changes which have improved with therapy, although, as yet, complete return to normal has not been obtained.

Findings in this study differ slightly from those previously reported in that the reticulocyte response to *L. casei* factor was much less than would have been anticipated with liver extract. Also the mild anemia of two patients with sprue was unaffected by pteroylglutamic acid, although improvement in other respects was noted. Liver extract, administered subsequently, was without effect on the blood picture of one of these patients but led to improvement in the other. Iron, in one instance, was not beneficial.

In previous studies *L. casei* factor has been administered only to patients in whom the bone marrow was hyperplastic and contained megaloblasts. In two patients in this series the bone marrow was depressed, and yet marked hematologic improvement occurred. In both instances, megaloblasts were the predominant cell of the hypoplastic marrow. Hematologic response also occurred with a cellular marrow in which pronormoblasts or normoblasts predominated. *L. casei* factor was not beneficial in aplastic anemia. Differentiation of anemias in which the bone marrow is depressed into those which will or will not respond to *L. casei* factor is difficult. If nutritional deficiency is present, or if megaloblasts are seen in the bone marrow, therapy with *L. casei* factor should be given a trial.

Eight patients have been treated continuously with *L. casei* factor for several months. In two the blood picture has become essentially normal (Patients 2 and 4). In the other six (Patients 1, 5 to 9) the hemogram has remained below the average normal,⁸ with erythrocytes varying from 3.9 to 4.3 million per cubic millimeter and the hemoglobin from 9.1 to 11.0 Gm. per 100 cubic centimeters. The volume of packed red blood cells has ranged from 36 to 41 per cent which is essentially normal. A deficiency of iron may account for some of these findings, and therapy with ferrous sulfate has been started in several patients. The amount of *L. casei* factor administered does not account for the subnormal blood picture, as seven of the eight patients received 30 mg. or more daily at some time during treatment. This large quantity does not appear to be more effective than 15 mg. daily. In all likelihood the smallest quantity required to produce a maximal response will be found to be less than

15 mg. daily in most patients, with an even smaller amount needed for maintenance. Several patients are being treated at the present time with 5 mg. daily.*

Very little difference has been noted between the response of patients to *L. casei* factor and to liver extract other than the finding of a smaller rise in reticulocytes with the former, in most instances, and the observation that *L. casei* factor is equally effective with oral or parenteral administration. The anemia of one patient with sprue in this series of cases responded to liver extract but not to *L. casei* factor. As Moore and associates² have indicated, the hemopoietic effect of liver extract cannot be attributed solely to the content of *L. casei* factor. Some extracts of liver contain very little, far less than enough to produce therapeutic effect. Also, crystalline material obtained from liver extract has produced maximal reticulocyte response in pernicious anemia in relapse when given in quantities of only 0.035 mg. daily.⁹ Thus, while pteroylglutamic acid and the erythrocyte maturing factor in liver extract produce similar effects, they appear to differ chemically and the mechanism of action of each requires elucidation.

SUMMARY

L. casei factor (pteroylglutamic acid) has been administered to fifteen patients with anemia in amounts of 5 to 120 mg. daily. Three patients with pernicious anemia and four with nutritional macrocytic anemia improved clinically and hematologically. There was a rise in reticulocytes and an increase in erythrocytes and hemoglobin which reached normal levels in two patients and remained slightly below normal in five. Leucocytes and platelets rose to normal in each instance in which initial values were low. In two patients who responded satisfactorily to pteroylglutamic acid, the bone marrow was depressed rather than hyperplastic and returned to normal within four to six weeks after therapy was instituted.

Two patients with sprue improved clinically, but the mild anemia which was present in each was unaffected by pteroylglutamic acid. The administration of liver extract failed to influence the anemia in one instance but was followed by improvement in the other. One child with a celiac syndrome was benefited slightly from both a clinical and hematologic standpoint when *L. casei* factor was administered.

L. casei factor was ineffectual in the treatment of two patients with aplastic anemia and of one patient each with macrocytic anemia associated with myxedema and regional ileitis and macrocytic anemia of unknown origin.

One patient with pernicious anemia and three patients with nutritional macrocytic anemia have been maintained in excellent condition for more than six months with pteroylglutamic acid as the therapeutic agent.

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*One patient with pernicious anemia in whom the initial erythrocyte count was 950,000 per cubic millimeter has shown a reticulocyte response of 42 per cent with 5 mg. pteroylglutamic acid daily.

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STUDIES IN IRON TRANSPORTATION AND METABOLISM

V. UTILIZATION OF INTRAVENOUSLY INJECTED RADIOACTIVE IRON FOR HEMOGLOBIN SYNTHESIS, AND AN EVALUATION OF THE RADIOACTIVE IRON METHOD FOR STUDYING IRON ABSORPTION

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THE data to be reported herein were obtained in a systematic study of the utilization of iron for hemoglobin synthesis. Observations were made both in human subjects and in dogs, under normal conditions and in the presence of various types of anemia. The method used was essentially that developed by Hahn and associates.¹ Tracer doses of radioactive iron (Fe^{59})* were injected intravenously, and the amount which later appeared in the peripheral blood as newly formed hemoglobin was measured.² In this way, both the rate and completeness of iron utilization were followed. It soon became apparent, however, that exact interpretation of results was frequently difficult because of variations in the rate of red blood cell formation and destruction. For instance, subjects with simple iron deficiency built all of the parenterally administered isotope into hemoglobin within six to nine days, while patients whose iron deficiency was associated with fever or a chronic debilitating disease used the iron more slowly and less completely. In these subjects, it was impossible to differentiate between an abnormality in iron utilization and some other disturbance in erythrocyte or hemoglobin formation.^{3, 4} Patients with hypoplastic anemia used very little of the injected radioiron for hemoglobin synthesis, presumably because they were forming few new red blood cells. This same relationship was even more clearly demonstrated in observations on patients with untreated pernicious anemia. Radioactive iron appeared slowly in the circulating hemoglobin of these subjects until liver extract was injected; its utilization was then sharply accelerated and eventually became complete. Increased rates of red blood cell destruction also influenced results. In patients with hemolytic anemia, the radioiron appeared very promptly in the circulating hemoglobin, but the concentration of radioactivity quickly leveled off at values usually much less than 100 per cent of the injected amount. Iron in these subjects was certainly being utilized at a rapid rate, and failure to recover all of the radioiron in circulating hemoglobin could not be interpreted as indicating poor utilization. As will be emphasized later, the results in hemolytic anemias are compatible with the thesis that recently stored iron is more easily mobilized than iron stored for longer periods and is drawn upon for daily needs.^{5, 6} When iron utilization is studied by the radioactive iron technique, therefore, several

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*Radioactive iron, the radioactive isotope of iron, and tagged iron are used as synonyms.

variables are involved. While interpretation of results is consequently more difficult and complicated, the results are of considerable interest.

In addition to providing fundamental information about both iron utilization and rates of hemoglobin synthesis, the results suggest that a re-evaluation should be made of the radioactive iron technique as a method of measuring iron absorption from the gastrointestinal tract. With this method, the amount of radioactive iron which appears in the total circulating blood within five to seven days after oral administration of the isotope is determined, and the ratio of this value to the total quantity of tagged iron ingested is taken as a measure of the amount absorbed.^{1, 7-12} Sufficient emphasis has not been given to the fact that the method measures only that portion of the absorbed iron which is utilized for hemoglobin synthesis. In experiments with normal and anemic dogs, sacrificed at varying times after radioiron had been fed, Hahn and co-workers¹ were able to demonstrate very little radioactive iron retained in the tissues. The assumption that iron utilized was equivalent to the amount absorbed was, therefore, thought to be a fair one. Results summarized in the preceding paragraph, however, suggest that the assumption is not valid when rates of red blood cell formation and destruction are disturbed. Other exceptions also have been described. Two growing puppies fed radioiron were found to have approximately three and four times as much of the isotope in their tissues as in their blood.⁸ They had been given the tagged iron during the course of twenty-seven days and had not been sacrificed until twelve days after the last dose. Utilization of the absorbed iron was so slow that a totally false impression would have been gained if the quantity which appeared in the blood as newly synthesized hemoglobin had been used as the only measure of absorption. Hahn and associates,¹⁴ furthermore, found that fourteen days after radioiron as ferrie ammonium citrate had been given intravenously to a normal dog the liver contained 82 per cent of the injected dose. These several considerations indicate clearly the need for an accurate definition of those circumstances in which iron utilization for hemoglobin synthesis is sufficiently complete that the radioactive technique can validly be used for measuring iron absorption.

For the sake of clarity, this paper will be divided into two parts. The first will present data on the utilization of injected radioiron; the second will relate this information to the radioactive iron method for studying absorption and will present additional pertinent data.

MATERIAL AND METHODS

The human subjects used were: (1) patients in the Barnes Hospital who volunteered as subjects and (2) healthy men, members of the house staff who had suffered no known blood loss and who had not recently served as donors for transfusions. The dogs were mongrels which had been kept in the animal quarters for at least a month, and usually much longer, before experimental observations were begun. During this time they were housed in individual cages, wormed, and fed Purina dog chow.

Hematologic techniques, methods for preparing samples and for measuring radioactivity,* were the same as those previously described.¹² The method of purifying the iron to free

*A metal Geiger counter tube with an aluminum window, supplied by the Physics Department of Washington University, has been used since 1945. This tube is less sensitive than the mica window counter which was used in the early part of this investigation but is more rugged.

it from radioactive cobalt and manganese, formed during the bombardment with deuterons in a cyclotron, has also been published in detail.¹² Radioactive ferrous ascorbate was prepared for intravenous injection from a stock radioactive ferric chloride solution as follows: Ferric hydroxide was precipitated by the addition of NaOH, the precipitate was spun down by centrifugation, and the supernatant liquid was discarded. Five parts of ascorbic acid to one part of iron were added to the precipitate in the centrifuge tube, and the mixture was stirred until the iron hydroxide dissolved. A few cubic centimeters of water were added, and the mixture was shaken vigorously for five minutes or until a purple solution was formed. This was poured into 20 volumes of acetone, the precipitated ferrous ascorbate was filtered off, and the salt was dried in a vacuum desiccator.¹³ Solutions of ferrous ascorbate are unstable and are decomposed by autoclaving. The salt, therefore, was kept in the dry state until immediately before it was to be injected. It was then dissolved in sterile 5 per cent glucose in normal saline and a measured amount was injected. A small portion of each solution was kept for determination of the actual iron content and for preparation of a standard for the radioactivity determinations. The doses of ferrous ascorbate used were small (less than 0.5 mg. per kilogram of body weight) and caused no reactions. A few subjects noted tingling at the site of the injection and a few detected a metallic taste. Dogs were frequently given ferric chloride instead of ferrous ascorbate.

To determine the radioactive iron in urine, feces, and tissues, it was necessary to separate the iron from phosphates by ether extraction. The material was wet-digested with sulfuric and perchloric acids, and the digest was made alkaline with sodium hydroxide. The copious precipitate of phosphates was dissolved in 6 N HCl and extracted with ether that had previously been saturated with 6 N HCl, by shaking in a separatory funnel. Two extractions were usually sufficient. In the case of urine and tissues low in iron, 1 or 2 mg. of inert iron as FeCl_3 were added to the digest as a carrier. The iron was recovered from the ether layer by shaking with normal HCl, the solution was warmed to evaporate the ether, and the iron was precipitated as the hydroxide and electroplated and counted as previously described.¹²

In the calculation of the percentage of the dose which appeared in the peripheral blood, the blood volume was arbitrarily assumed to be 80 c.c. per kilogram. This assumption was probably no more inaccurate than would have been the estimation of red blood cell mass from determination of blood volume made by standard dye procedures. Correction was made for the iron removed in sampling.

I. Utilization of Intravenously Administered Radioactive Iron

A. Healthy Human Subjects and Normal Dogs.—Radioactive ferrous ascorbate was given intravenously in single doses which provided 9 to 18 mg. of the metal to four healthy young men, only one of whom had served as a donor within the previous year. The rate and completeness of iron utilization are illustrated in Fig. 1; hematologic data are recorded at the bottom of the chart. By the sixth day, 60 to 70 per cent of the injected iron had appeared in the peripheral blood; after this first rapid utilization, the amount increased slowly until 80 per cent or more was present at the end of seven to ten weeks. Positive errors in estimating blood volume probably account for the occasional values above 100 per cent. The prompt utilization during the first week is of considerable interest. If the injected iron had been added to the whole of the body's store of nonhemoglobin iron and had been used at the same rate as the stored iron was being mobilized, then it would have appeared much more slowly as newly synthesized hemoglobin. The result would seem to indicate that newly

injected iron is selectively used, initially at least, for new hemoglobin formation. Iron which has been stored for a longer period of time may not be so readily mobilized.

In normal dogs, parenterally administered radioiron was used less completely and somewhat more slowly than in the healthy human subjects. From 0.2 to 9.0 mg. of labeled iron, as ferric chloride or as ferrous ascorbate, were injected into thirteen animals. Results are charted in Fig. 2; the striated area delineates the extreme values obtained from the four healthy human subjects previously described and is included to make comparison easier. Even though

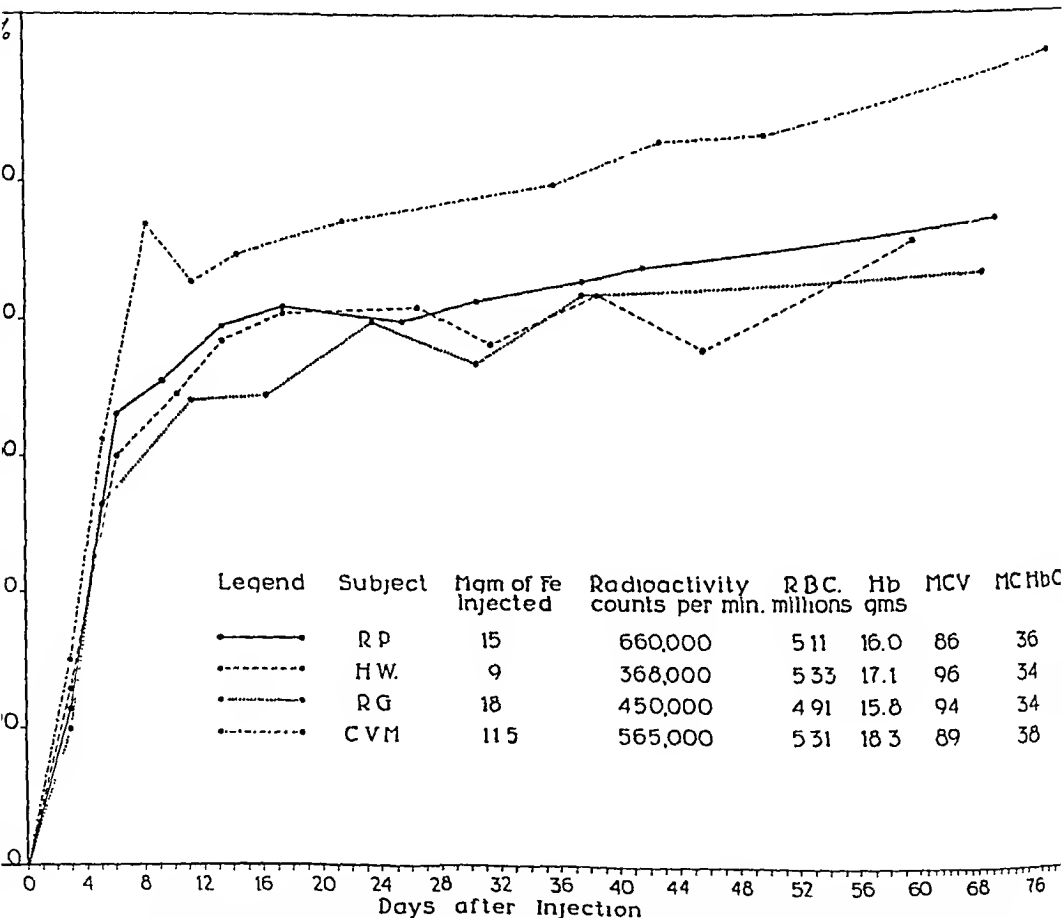


Fig. 1.—The utilization of radioactive iron by normal men. The iron was given intravenously as ferrous ascorbate in the following doses: R. P., 0.19 mg. iron per kilogram of body weight; H. W., 0.12 mg. per kilogram; R. G., 0.21 mg. per kilogram; C. V. M., 0.13 mg. per kilogram.

observations were continued in several instances for sixty days or more, the total amount of the isotope which eventually appeared in the peripheral blood of these animals varied from 36 to 72 per cent of the amount injected, and in no instance was utilization as complete as in the human subjects. In only six of the dogs was 60 per cent or more of the injected dose found in the peripheral

blood at any one time. Completeness of utilization was not closely correlated with the size of the dose.* The animal (Dog 19) which received 9.0 mg. per kilogram body weight, for instance, used 45 per cent, while Dogs 55 and 58 given less than 1 mg. per kilogram also utilized only 47 and 45 per cent. On the other hand, the three dogs with the greatest utilization (Dogs 25, 26, 42) received 0.5 mg., 1.6 mg., and 5.0 mg. per kilogram, respectively. It should be emphasized, however, that even the larger amounts given were only tracer doses.

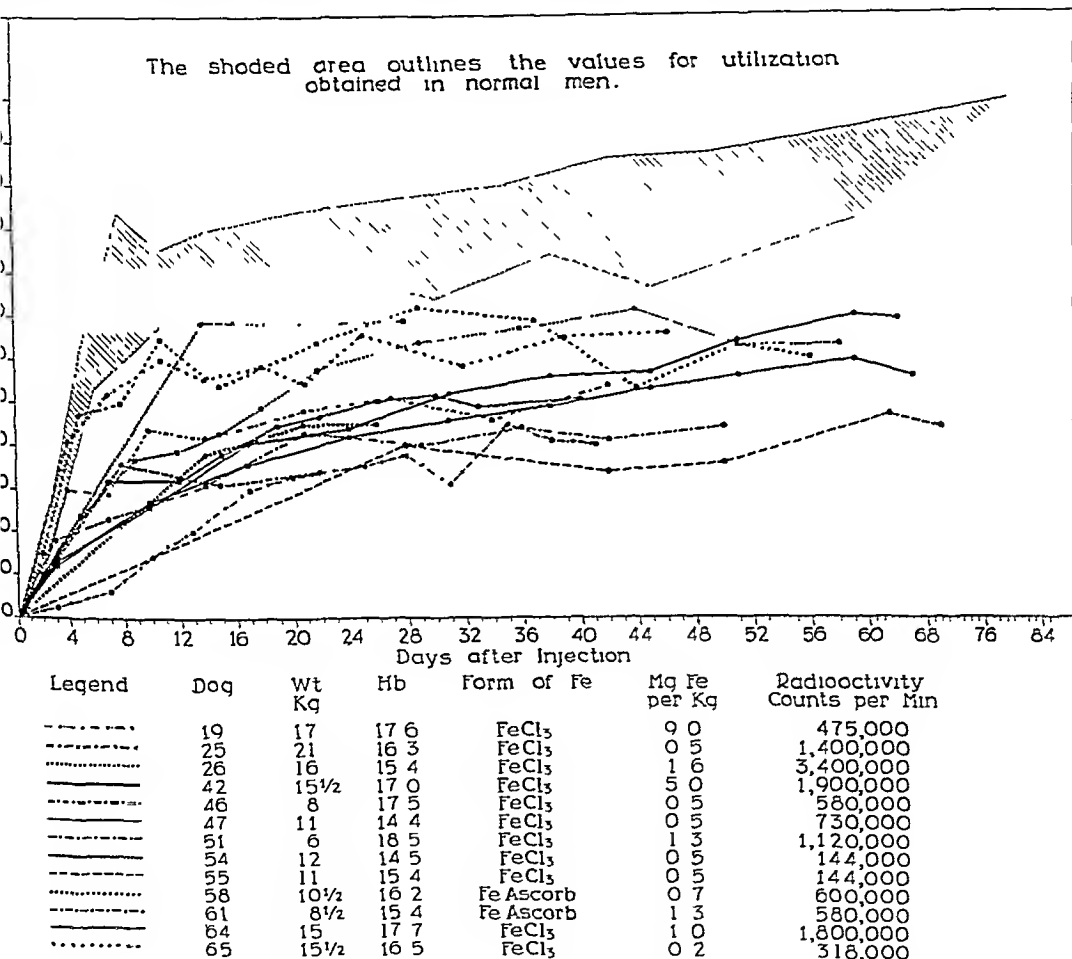


Fig. 2.—Appearance of radioactive iron in the blood of normal dogs after intravenous injection.

Attempts were made to find out what happens to the radioiron which does not appear in the peripheral blood as hemoglobin. Two animals (Dogs 58 and 51), in which the level of circulating radioactive iron had remained relatively constant for several weeks, were perfused for two hours under nembutal anesthesia with Locke's solution. The amount of the isotope which appeared in the

*Calculated in terms of the per cent of the administered dose, the rate of iron utilization was roughly proportional to the per kilogram size of the dose. With the larger amounts injected, however, more actual milligrams of iron were built into hemoglobin during the first week than with the smaller quantities.

perfusate and tissues was then determined. In Dog 58, the weights of the bone marrow, muscle, and skin were estimated, but in the other animal, a more complete sampling was obtained. All of the muscle was carefully cut from the body, ground, weighed, and an aliquot of the mixed tissue was taken for analysis. The whole skeleton was dry ashed, the ash was dissolved in 6 N HCl, and aliquots were taken for the radioiron determinations. The entire skin was removed and dry ashed; radioactivity was determined in HCl aliquots of the ash. In addition, all the radioiron excreted in the urine and feces of Dog 51 was measured for the whole fifty-day period; 8 per cent of the injected dose was recovered in the excreta. Half this amount was recovered in the urine within a few hours after injection of the isotope; however, after this brief period only small amounts appeared in the urine, and almost all of the additional excretion was by way of the intestinal tract. Results are recorded in Table I. The total amount of radioiron recovered in the tissues of these two dogs amounted to 17.1 and 41.6 per cent of the injected dose, but even so all of the iron could not be accounted for. If Dog 58 excreted as much of the dose as Dog 51, the total recovery would still be only 86 and 72 per cent, respectively. Hahn and associates¹ had a similar experience. The "lost iron" probably reflects the inadequacies of the method for extracting all of the iron from tissues and for measuring the isotope when amounts of radioactivity are small.

TABLE I. RADIOACTIVE IRON FOUND IN TISSUES OF TWO DOGS TWENTY-SIX AND FIFTY DAYS AFTER IRON HAD BEEN INJECTED INTRAVENOUSLY

	DOG 58	DOG 51
Weight (kg.)	10.5	6
Hematocrit	47	56
Hemoglobin (Gm.)	16.8	18.5
Radioiron injected (mg.)	7.3	7.5
	(Ferrous ascorbate)	(FeCl ₃)
Time sacrificed after I.V. iron (days)	26	50
Radioiron found in:	% of amount injected	
Blood and perfusion fluid	35.5	42.2
Bone marrow	10.3*	1.8
Liver	23.8	8.4
Spleen	1.0	0.3
Kidneys	1.1	0.6
Muscle	4.2*	3.3
Skin	1.2*	1.9
All other tissues	---	0.9
Excreted during experiment	---	8.3
Removed by sampling	0.9	4.2
Total radioiron accounted for	78.0	71.9

of measurement. It has, however, become a common practice in the literature, and we have consequently recorded our data to the first decimal place.

*Estimated weight of bone marrow was 300 Gm.; muscle, 3,000 Gm.; skin, 900 Gm.

In an additional attempt to recover all of the injected radioactive iron, three normal animals whose circulating hemoglobin level of radioiron had stabilized were bled several times a week for six to eight weeks. Two to three hundred cubic centimeters of blood were removed at each bleeding, and the total amount of radioactive iron withdrawn was measured (Table II). In two dogs, 16 and 28 per cent more of the injected isotope was removed in this manner than had

TABLE 11. ATTEMPTS TO PULL RADIOIRON FROM TISSUES OF NORMAL DOGS BY SYSTEMATIC BLEEDINGS

TABLE 11. ATTEMPTS TO OBTAIN RADIOIRON

DOG	WEIGHT (KG.)	ES- TIMATED BLOOD VOLUME (C.C.)	HEMO- GLOBIN (GM.)	HEMATO- CRIT	RADIOIRON INJECTED (MG.)	RADIOIRON IN BLOOD VOLUME			RADIOIRON REMOVED BY BLEEDING			PULLED OUT OF TISSUES	EX- CRETED	% IN BLOOD AT END OF EXPERI- MENT	% OF DOSE AC- COUNTED FOR
						DAY	% OF DOSE	O.C. OF BLOOD	DAY	% OF DOSE					
61	8.6	690	17.7	51	11.6 (Ferrous ascorbate)	8	35.6		30 to 40	896	37.0				
						15	30.9		41 to 50	654	14.9				
						22	34.0		51 to 60	631	8.1				
						30	38.2		61 to 70	740	4.2				
									71 to 77	490	1.3				
61	15	1,200	16.3	46	16.0 (FeCl ₃)				106	285	0.5				72.0
									Total removed		66.0			27.8	
						26	50.0		0 to 40	52	1.7†				
						30	51.0		41 to 50	755	27.1				
						33	48.4		51 to 60	550	15.3				
						40	50.2		61 to 70	854	13.4				
						134	1.4		71 to 80	830	6.0				
									81 to 90	768	2.1				
									91 to 95	275	0.5				
									134	306	0.4				
65	15.5	1,240	16.5	47	3.2 (FeCl ₃)				Total removed		66.5			16.3	67.9
						25	66.5		0 to 40	95	4.4†				
						32	59.0		41 to 50	533	21.7				
						39	64.6		51 to 60	757	18.3				
						46	66.5		61 to 70	585	10.2				
65	15.5	1,240	16.5	47	3.2 (FeCl ₃)	134	5.4		71 to 80	900	6.6				74.3
									81 to 85	550	3.1				
									134	298	1.6				
									Total removed		68.9			2.4	

*Excreted in urine and stool in first three days.

†Removed in ten small samplings.

appeared at any one time previously in the circulating hemoglobin. These results also demonstrate that normal dogs do not regularly utilize injected radioactive iron completely for hemoglobin synthesis and at the same time show that the portion which had been stored is available for hemoglobin formation when the demand for iron becomes great enough. However, there still remained a fairly large amount (20 to 25 per cent) of the injected dose which was not accounted for, even if it be assumed that 10 per cent had been excreted during the period of observation.

B. Iron-Deficient Patients and Dogs.—Tracer doses of radioactive ferrous ascorbate were given intravenously to four patients with hypochromic microcytic anemia. As may be seen from Fig. 3, the rate of utilization was slightly more rapid than it had been in the normal controls. The amounts of iron

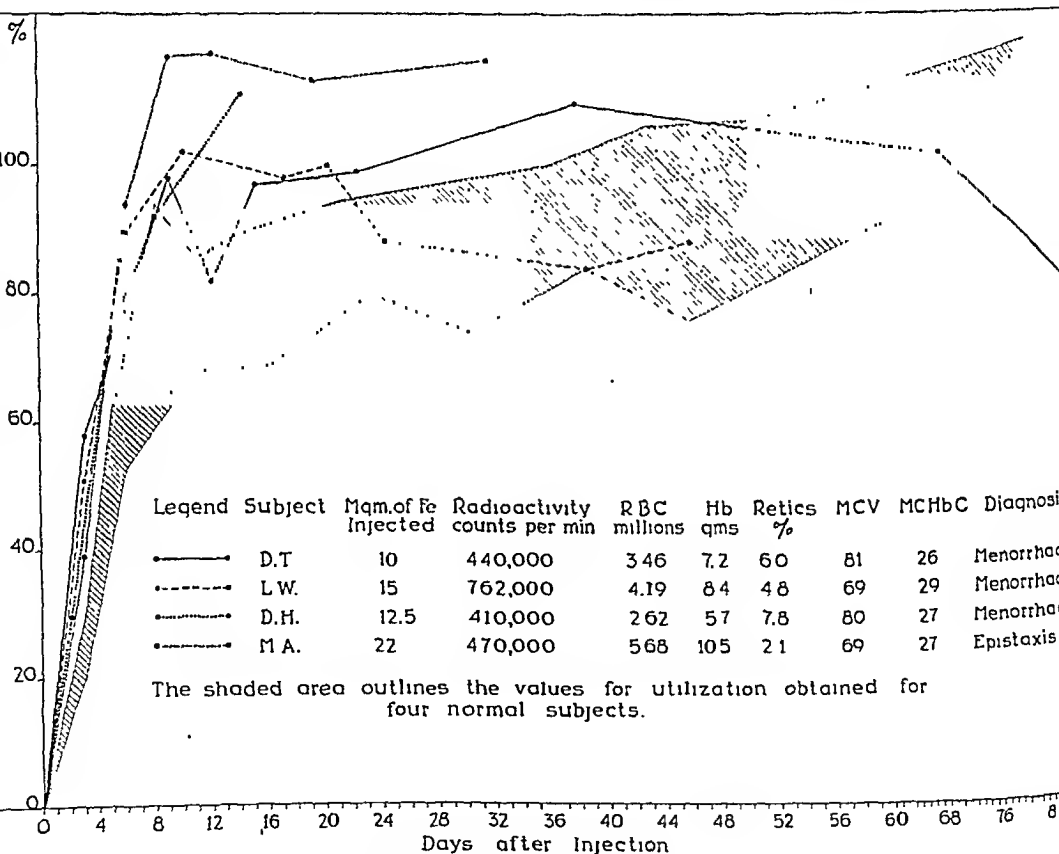
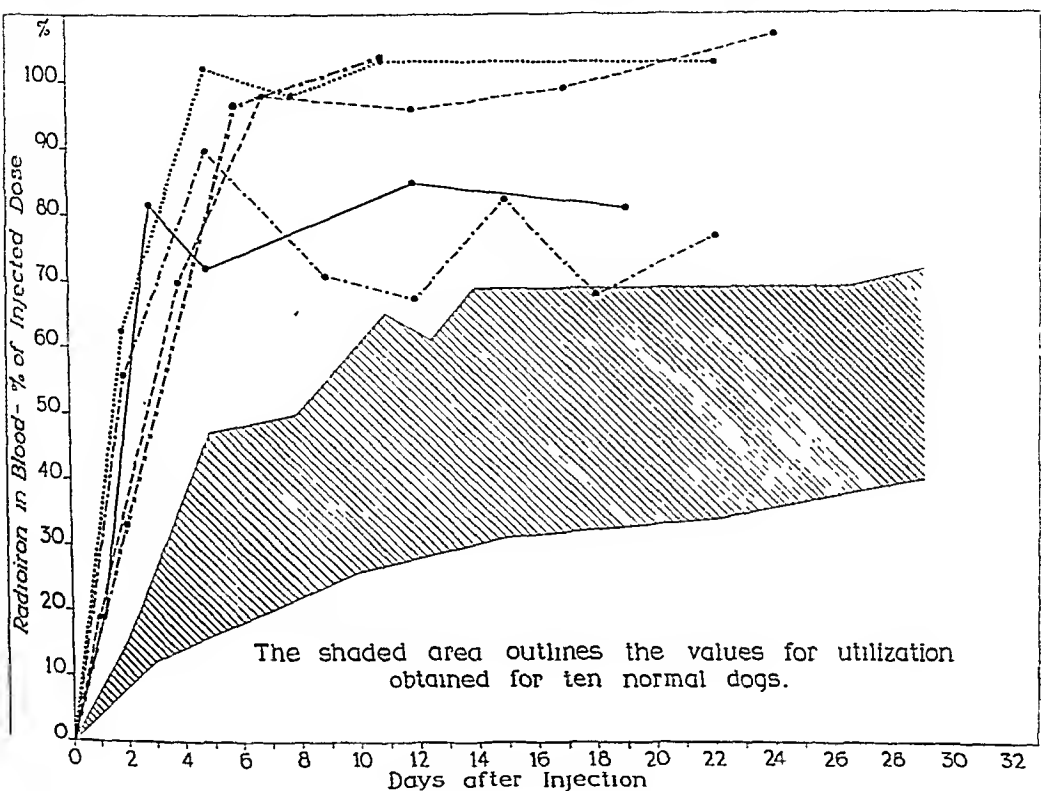


Fig. 3.—The utilization of radioactive iron by patients with hypochromic anemia. Iron given intravenously as ferrous ascorbate.

injected, the radioactivity, and the hematologic data for each individual are tabulated at the bottom of the illustration. In all four subjects, utilization of the injected iron was complete by the tenth day.

Iron-deficient dogs also promptly utilize 80 per cent or more of injected radioactive iron for hemoglobin synthesis (Fig. 4). Each of the five dogs was

prepared by withdrawing about 20 to 25 per cent of its blood volume two or three times a week for a period of two to five months; during this time the animals were given a diet of bread and milk so that the iron intake would be limited and the induced deficiency be more complete. A vitamin B supplement

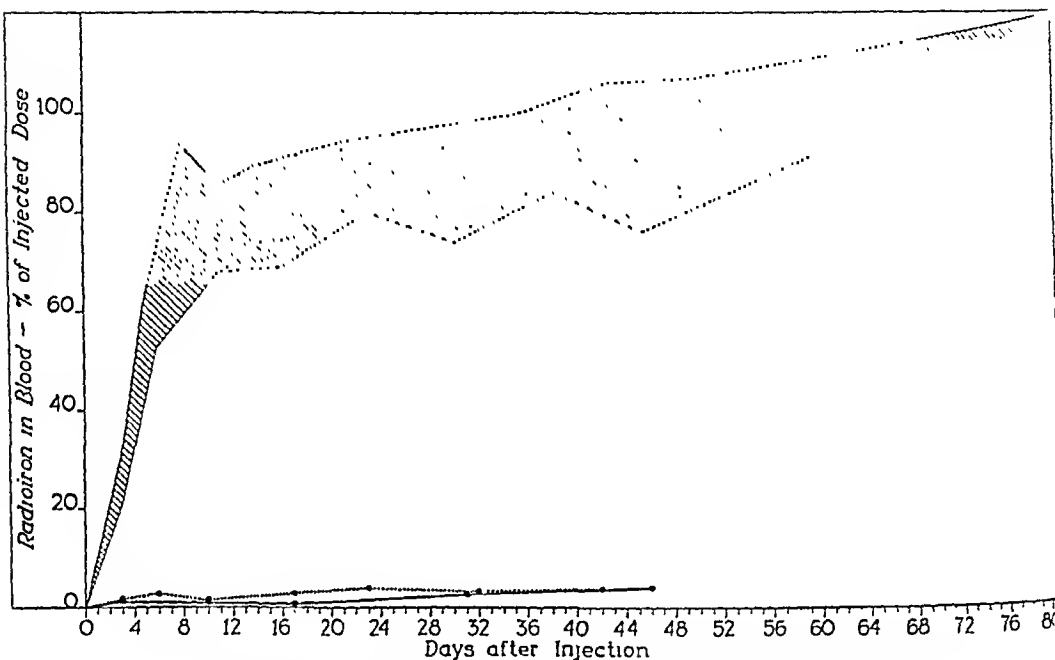


Legend	Dog	Wt Kg.	Experiment	Hb	Mg Fe per Kg.	Form of Fe Injected	Radioactivity Counts per Min
.....	23	8.4	Anemic from hemorrhage	5.6	1.6	FeCl ₃	1,800,000
.....	24	16.4	Anemic from hemorrhage	5.6	0.5	FeCl ₃	1,130,000
.....	18	20.4	Anemic, massive dose of Fe; then R.I	7.4	13.0	Fe(OH) ₃ ; then FeCl ₃	2,000,000
.....	21	13.2	Anemic, massive dose of Fe; then R.I	5.7	26.0	Fe(OH) ₃ ; then FeCl ₃	2,100,000
.....	63	11.6	Anemic from hemorrhage	6.6	1.3	FeCl ₃	204,000

Fig. 4.—The appearance of intravenously injected radioactive iron in the blood of five dogs which had been made anemic by chronic hemorrhage.

was regularly administered. The hemoglobin levels of the animals at the time radioactive iron was injected, with other pertinent data, are given at the bottom of the chart. Two of the animals (Dogs 18 and 21) were given 254 and 337 mg. of iron as colloidal ferrie hydroxide intravenously shortly before the tracer

doses of radioiron were injected. These amounts provided enough iron to raise the hemoglobin from the existing levels of 5 to 7 Gm. to a value of 14 grams. It was thought that the administration of these relatively large amounts of inert metal might slow the utilization of the isotope, but the radioactive iron appeared as rapidly and almost as completely in the circulating hemoglobin as it had in the other three animals. From 70 to 90 per cent of the injected doses was utilized for hemoglobin formation.



Legend	Subject	Mgm. of Fe Injected	Radioactivity counts per min.	R.B.C. millions	Hb gms	Retics %	MCV	MCHbc	Diagnosis
—●—	J.S.	18	500,000	415	13.3	0	94	34	Hypoplastic Anemia
—●—	E.H.	13	444,000	3.26	9.9	0.2	93	32	Hypoplastic Anemia

The shaded area outlines the values for utilization obtained for four normal subjects.

Fig. 5.—The appearance of radioactive iron in the blood of patients with hypoplastic anemia after intravenous injection of ferrous ascorbate.

C. Patients With Hypoplastic Anemia.—Radioactive ferrous ascorbate was injected intravenously into two patients with hypoplastic or refractory anemia. In both subjects, the blood platelet level was high enough to prevent any hemorrhagic manifestations. At the time of the injection, the erythrocyte counts had been raised to 4.15 and 3.26 million cells per cubic millimeter by previous transfusions. Additional blood had to be administered at intervals of two to three weeks during the course of the observations in order to prevent the red blood cell level from falling too low. The bone marrow showed very few erythroid elements, and reticulocytes were rare. In neither patient did as much as 4 per cent of the injected iron appear in the blood as hemoglobin during six weeks of observation (Fig. 5). This result is what would have been predicted.

If very little hemoglobin is being formed, there is little opportunity for the radioactive iron to be built into hemoglobin.

D. *Patients With Addisonian Pernicious Anemia.*—Study of the rate at which two patients with pernicious anemia in relapse utilized iron proved particularly interesting. For two weeks after the radioiron had been injected,

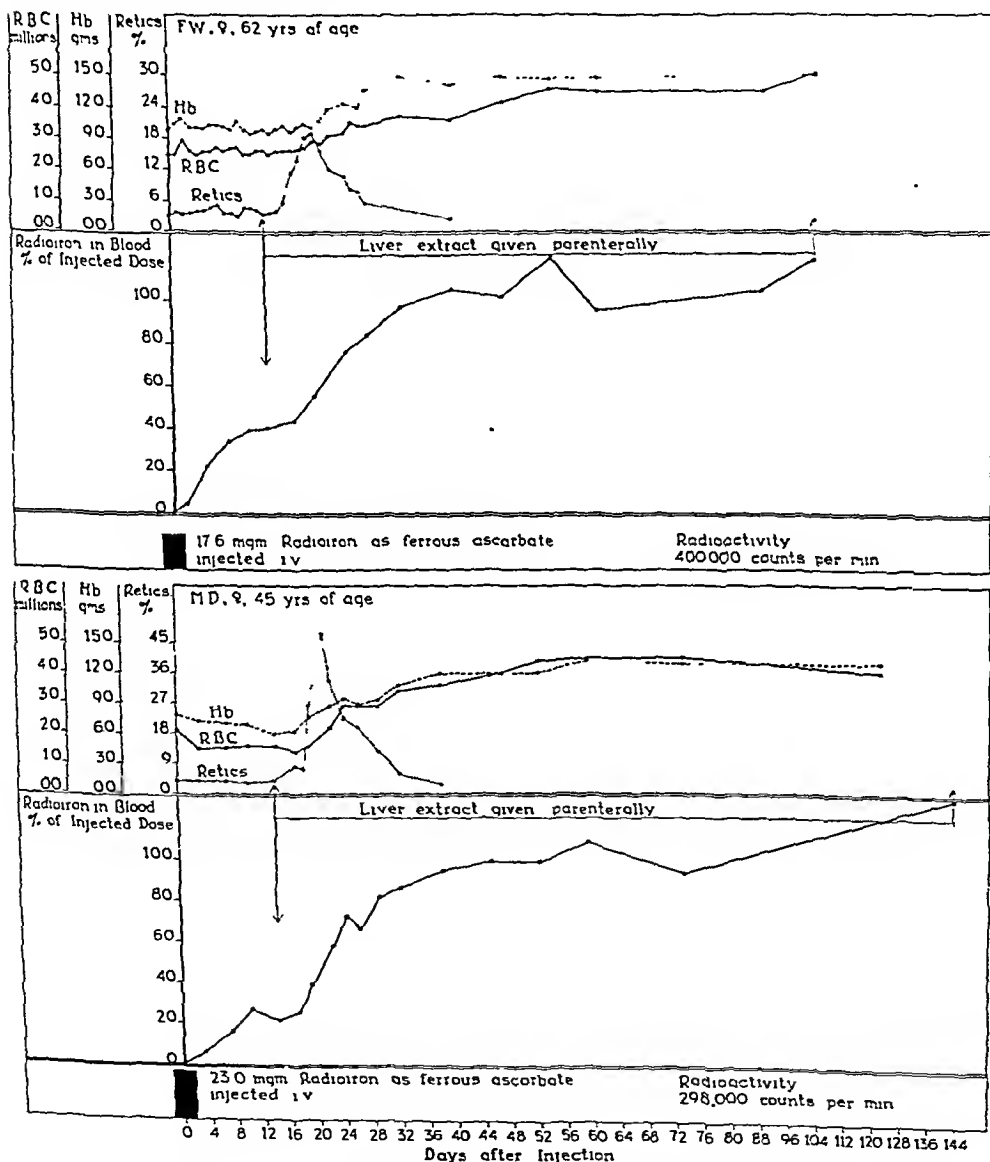


Fig. 6—The utilization of radioactive iron by two patients with pernicious anemia. Iron given intravenously as ferrous ascorbate.

therapy with liver extract was withheld, and the patients were fed a meat-free diet. The iron appeared rather slowly in the circulating hemoglobin and then tended to level off at about 20 and 40 per cent of the injected dose (Fig. 6).

Clinical circumstances demanded that specific therapy be started without additional delay, and the administration of liver extract was begun on the fifteenth day in each instance. Coincident with the reticulocyte rise, there was a sharp increase in the circulating radioiron, and within two to three weeks all of the isotope had been synthesized into hemoglobin. Observations were continued for three to five months; during this period the level of radioiron in the peripheral blood remained high. The initial slow rate of utilization was probably a

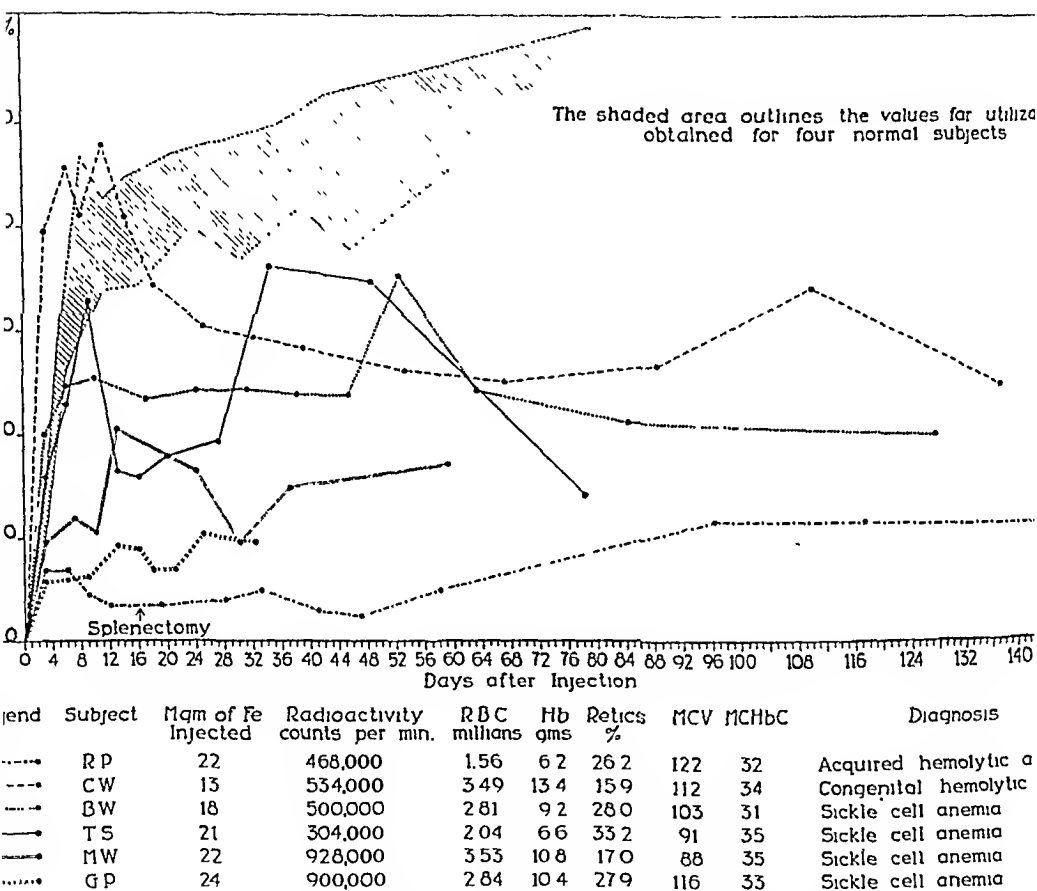


Fig. 7.—The appearance of radioactive iron in the blood of patients with hemolytic anemias after intravenous injection of ferrous ascorbate.

reflection of the relative erythroid maturation arrest which exists in patients with untreated pernicious anemia. As soon as red cell maturation was stimulated by liver extract, the rate at which new hemoglobin was delivered to the peripheral blood was accelerated, and the injected radioactive iron was utilized more rapidly.

E. Hemolytic Anemias in Patients and Dogs.—The effect of hemolytic anemias on the appearance of injected radioiron in the blood was studied in six patients: one with acquired hemolytic anemia, one with congenital hemolytic

anemia, and four with sickle-cell anemia.* Results are graphically recorded in Fig. 7. The initial rate of utilization was rapid in each instance but the rise usually ended abruptly, and in two of the patients the curves formed a plateau at less than 20 per cent of the dose. A value of over 90 per cent was reached in only one subject. It is suggested that because of the hemolytic process and the resultant rather short length of life of the red cells, many of the erythrocytes which contain radioactive iron in their hemoglobin are destroyed within a few days after their delivery to the peripheral blood. Variations in the rate of hemolysis probably account in large part for the different amounts of radioiron found in circulating hemoglobin at any one time. There were, furthermore, wide variations in the level of radioiron present in the same subject from week to week. These fluctuations are in decided contrast to the relatively smooth curves obtained in other patients and can probably also be explained on the basis of variations in the equilibrium between erythrocyte formation and destruction. A splenectomy was performed on the patient with acquired hemolytic anemia, and the excised spleen was found to contain 35 per cent of the radioiron that had been injected. After splenectomy, the patient's red blood cell count rose slowly to normal. With this improvement, the amount of circulating radioiron increased from 8.4 to 23.5 per cent of the injected dose. There are three probable reasons why a larger amount of the iron did not ultimately appear in the blood: (1) One-third of the injected dose had been removed in the spleen. (2) Some of the isotope may have been stored elsewhere. (3) Excretion of radioiron was probably significant, since it has been shown by Hahn and co-workers¹⁶ that in dogs with hemolytic anemia induced by phenylhydrazine excretion is several times greater than normal.

Hemolytic anemia was induced in dogs by the oral administration of phenylhydrazine. All of the animals initially had hemoglobin levels above 15 Gm. and erythrocyte counts greater than 6.0 million cells. In Dogs 56, 60, and 59 (see Table III), the radioactive iron was given nineteen, twenty-nine and eighty-five days, respectively, after phenylhydrazine administration had been started, and the drug was continued for sixteen to twenty-eight additional days. The hemolytic anemia was maintained for two to four weeks after the radioiron had been injected, although the dose of phenylhydrazine was not regulated well

*Since this manuscript was submitted for publication, radioiron has been given intravenously to a seventh patient with hemolytic anemia. This patient was a white man, 58 years old, with an acquired hemolytic anemia; his red blood cell count was 1.3 million, and 36 per cent of his erythrocytes were reticulocytes. Fourteen milligrams of radioactive iron were given intravenously as ferrous ascorbate (0.19 mg per kilogram of body weight). Three days later 20 per cent of the injected dose had appeared in the peripheral blood. Two days later the patient became acutely ill; he died on the seventh day after the injection. On the day of death the radioiron in the blood had dropped to 5.9 per cent. Assay of tissues obtained at necropsy gave the following results.

TISSUE	WEIGHT (GM.)	% OF INJECTED RADIOIRON
Spleen	630	19
Liver	2,540	40
Kidneys	430	1
Lungs	1,140	1
Bone marrow	3,360 (Estimated)	1
		Total found 82

Complete stool collections were made from the time the radioiron was injected until death. In spite of the intensity of the hemolytic process (17 Gm. of urobilinogen were excreted in the feces in three days), only 0.06 per cent of the injected radioiron appeared in the stools. Within four hours of the injection 1.3 per cent of the injected iron appeared in the urine. Of the injected dose, then, 1 per cent spilled over in the urine, 6 per cent was found in the red cells on the day of death, and 82 per cent was estimated to be in the tissues examined. Eleven per cent was not accounted for.

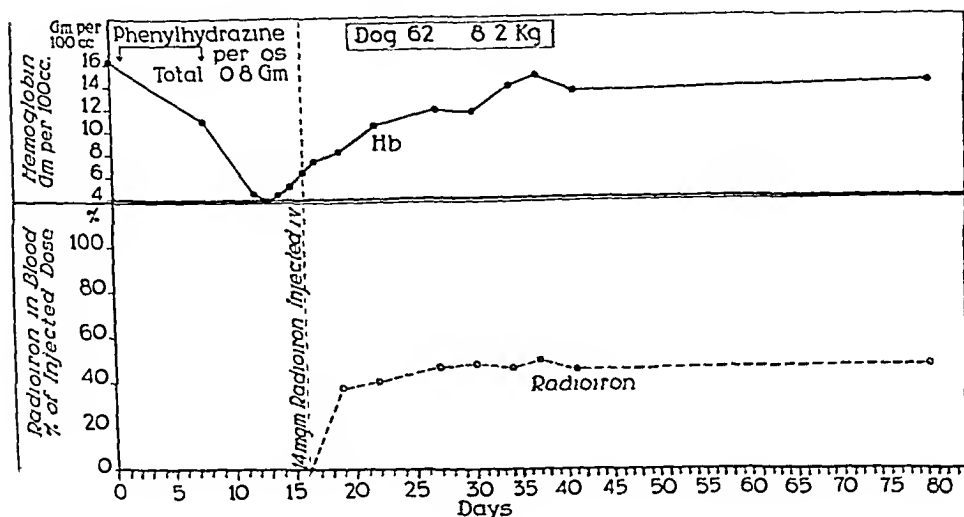
TABLE III. APPEARANCE OF RADIOIRON IN BLOOD OF DOGS WITH HEMOLYTIC ANEMIA INDUCED BY PHENYLHYDRAZINE

	DOG 29	DOG 56	DOG 59	DOG 60	DOG 62
Weight (kg.)	17	11.0	9.1	6.2	8.2
Initial hematologic data:					
R.B.C. (millions)	7.70	6.86	7.30	6.78	6.37
Hemoglobin (Gm./100 c.c.)	19.2	16.5	16.4	15.6	16.3
Phenylhydrazine (total days given)	39	35	113	49	7
Radioactive iron injected (I.V.):					
Days after start of phenylhydrazine	40	19	85	29	13
Milligrams per kilogram	0.5	1.0	1.1	1.7	1.4
Counts per minute in total dose	5,450,000	601,800	960,000	176,000	280,000
R.B.C. on day iron injected (millions)	2.95	2.55	2.21	1.39	1.83
Hemoglobin on day iron injected (Gm.)	9.4	6.5	4.9	3.3	6.6
Reticulocytes on day iron injected (%)	40.6	34.1	---	---	18.6
Time phenylhydrazine was discontinued:					
Days after injection of radioiron	---	16	28	20	---
Days before injection of radioiron	1	---	---	---	6
Maximum level of radioiron which appeared in blood (% of total dose)	66.5	46.9	30.5	52.6	49.4
R.B.C. at time of maximum level of radioiron	6.00	6.65	6.62	4.62	5.87
Hemoglobin at time of maximum level of radioiron	16.2	15.0	13.6	11.3	14.8
Radioiron removed by sampling and subsequent bleedings (% of dose)		---	---	51.1	53.5
Radioiron recovered in perfusate and blood, tissues, and in excreta collected in three weeks		70.1*			

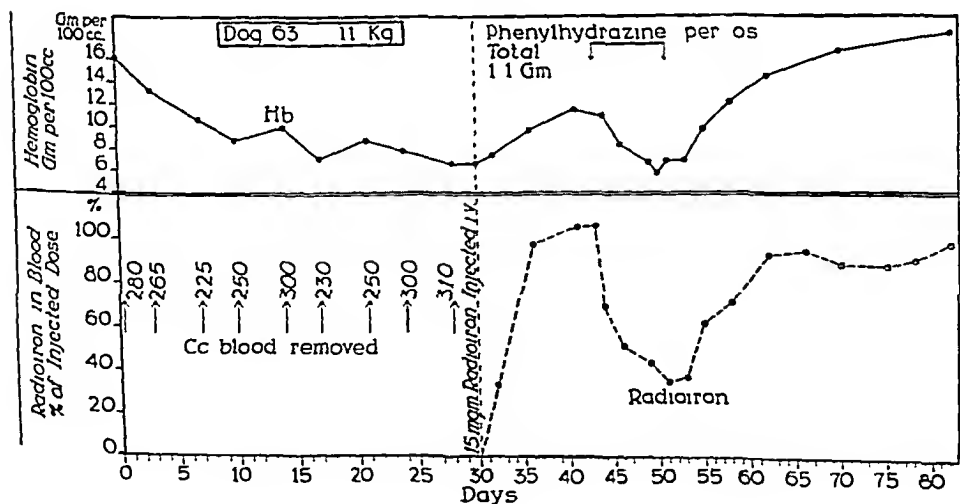
*Of this amount, 32.7 per cent was in blood and perfusate; 25.3 per cent was in the tissues; 6.5 per cent was recovered in urine and stools; and 2.6 per cent had been removed by sampling.

enough to keep the red blood cell and hemoglobin levels constant. The isotope appeared promptly in the peripheral blood, but in the first week only 13 to 22 per cent of the injected dose was demonstrated in the circulating hemoglobin. The levels stabilized at approximately these values until the phenylhydrazine was discontinued and the red cell count began to rise. The maximum amounts of radioiron which appeared in the blood of these three dogs, however, were 30.5, 46.9, and 52.6 per cent, respectively, in spite of the fact that the hemoglobin values rose sharply during the postphenylhydrazine period. It seemed possible that a greater proportion of the isotope might appear in circulating hemoglobin if the iron were injected after phenylhydrazine administration had been stopped, that is, at a time when the red blood cell count was definitely increasing. Accordingly, Dogs 29 and 62 were given the iron one and six days, respectively, after the last dose of phenylhydrazine (see Table III and Fig. 8). The maximum amounts of radioiron found in the blood of these two animals were 66.5 and 49.4 per cent, respectively, of the quantities injected; these values do not differ significantly from those obtained with the other three animals. To see whether the radioiron which had not appeared in the circulating hemoglobin was in a form which could be used for hemoglobin synthesis, two animals (Dogs 60 and 62) were subjected to massive bleedings for a period of weeks, and the total amount of isotope removed was determined. One of the animals had received the intravenous injection of iron before and the other after discontinuance of phenylhydrazine. In neither instance did the amount of iron

recovered by the bleedings differ significantly from that calculated as being present in the blood stream at the time the systematic venesections were begun (Table III). It seemed unlikely that all of the radioiron unaccounted for had been excreted, even though excretion in the presence of hemolysis may be greater



A.



B.

Fig. 8.—A, The effect of a hemolytic anemia (phenylhydrazine) on the utilization of injected radioiron by a normal dog. B, The effect of a hemolytic anemia (phenylhydrazine) on the level of injected radioiron in the blood of an iron-deficient dog.

than normal.¹⁶ To make certain, however, the excretion in urine and feces during three weeks of observation was measured in Dog 56, which had received phenylhydrazine for sixteen days following injection of the iron. Only 6.5 per cent of the dose could be accounted for in this manner. On the fiftieth day, the animal was anesthetized with nembutal, perfused, and sacrificed. The total

amount recovered in the perfusate, tissues, and excreta amounted to 70.1 per cent of the dose; 28.3 per cent was found in the tissues.

These results are not necessarily at variance with those reported by Cruz and associates.¹⁷ The latter authors demonstrated that radioiron released from the hemoglobin of hemolyzed red cells could be reutilized for hemoglobin synthesis. Their published data, however, do not show what per cent of the injected dose was found in the peripheral blood at any one time.

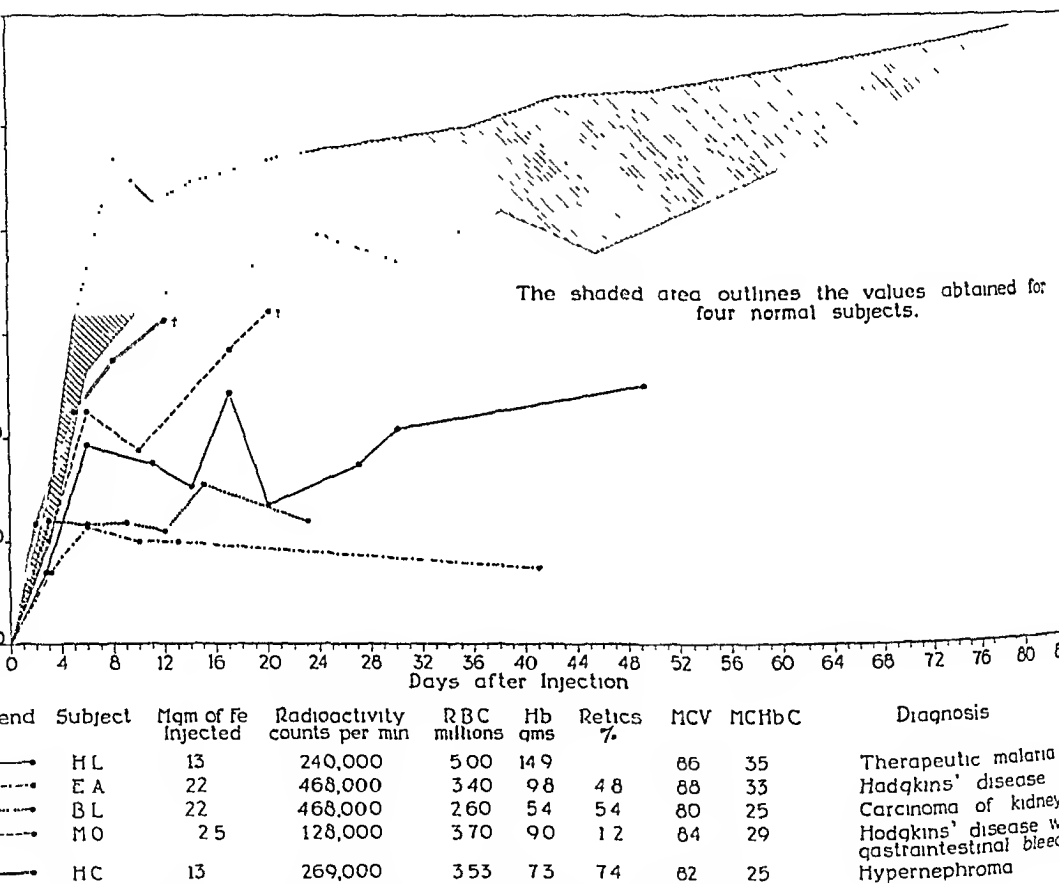


Fig. 9.—The appearance of radioactive iron in the blood of patients with fever after intravenous injection of ferrous ascorbate.

One other animal (Dog. 63) was partially depleted of its iron reserves, given radioiron intravenously, and permitted to utilize the injected iron completely for new hemoglobin before phenylhydrazine was administered (Fig. 8). With the development of the phenylhydrazine anemia, the circulating radioiron decreased to 35 per cent. When phenylhydrazine was discontinued and the animal was permitted to recover from its anemia, 98 per cent of the radioiron again appeared in the blood stream as circulating hemoglobin. This observation confirms Cruz's evidence that the radioiron released from liberated hemoglobin can be reutilized. When it is appraised in comparison with results ob-

tained on normal animals, however, it indicates that the normal dogs had such a plentiful supply of iron in their pools of iron available for hemoglobin synthesis^{5, 6} that they used only a fraction of the tagged element.

F. Patients With Fever or Chronic Disease.—There is convincing evidence to indicate that iron utilization is impaired in the presence of fever or chronic disease.^{3, 4, 15} The extent to which utilization is prevented, however, is not known. To obtain data in partial answer to this question, radioactive ferrous ascorbate was injected intravenously into five subjects: one with therapeutic malaria, two with Hodgkin's disease, and two with malignant tumors (Fig. 9). Three of the patients had low mean corpuscular hemoglobin concentrations; they were, then, iron deficient. In spite of this added stimulus to iron utilization, none of the subjects developed concentrations of circulating radioiron greater than 62 per cent of the dose, and in two the maximum amounts were less than 30 per cent.

II. Limitations of the Radioactive Iron Method for Measuring Iron Absorption From the Gastrointestinal Tract

From the observations presented in the preceding section, it is evident that not all of the radioactive iron introduced into the body necessarily finds its way to the peripheral blood as newly synthesized hemoglobin. Therefore, if that fraction of the oral dose of radioiron which appears in circulating hemoglobin is taken as the measure of absorption, the resultant values will frequently be low. Erroneous conclusions may, consequently, be drawn as to the ability of the animal organism to absorb iron under a variety of conditions. There is reason to believe that iron absorbed from the intestinal tract is used for hemoglobin synthesis in the same manner as is iron injected intravenously. If this is true, then, on the basis of the results described, one can make the following statements as to the adequacy of the radioactive iron technique for measuring iron absorption:

1. In subjects with uncomplicated iron deficiency, the method accurately measures absorption.
2. In normal adult men, the method also provides reasonably accurate values. The amount of radioactive iron which appears in circulating hemoglobin within six to nine days, however, may represent only 70 to 90 per cent of the dose, and further slight increases may occur during the subsequent three or four weeks. In healthy dogs, the circulating radioactive hemoglobin iron may be only 35 to 70 per cent of the iron retained.
3. In hypoplastic anemias and in untreated pernicious anemia, because of the slow rate of erythrocytogenesis, the amount of iron built into hemoglobin cannot be accepted as a measure of iron absorption.
4. In hemolytic anemias, the amount of radioactive iron built into hemoglobin and found in the peripheral blood at any one time may be anywhere from 10 to 90 per cent of that actually absorbed.

5. In patients with fever caused either by infection or other disorders, only 20 to 60 per cent of the absorbed radioiron may appear in circulating hemoglobin.

An excellent opportunity to test the validity of our concern about the method is provided by patients with pernicious anemia in whom erythrocytogenesis can be stimulated by injections of liver extract. Accordingly, the following observations were made: To E. G., a white man, 47 years of age, who had

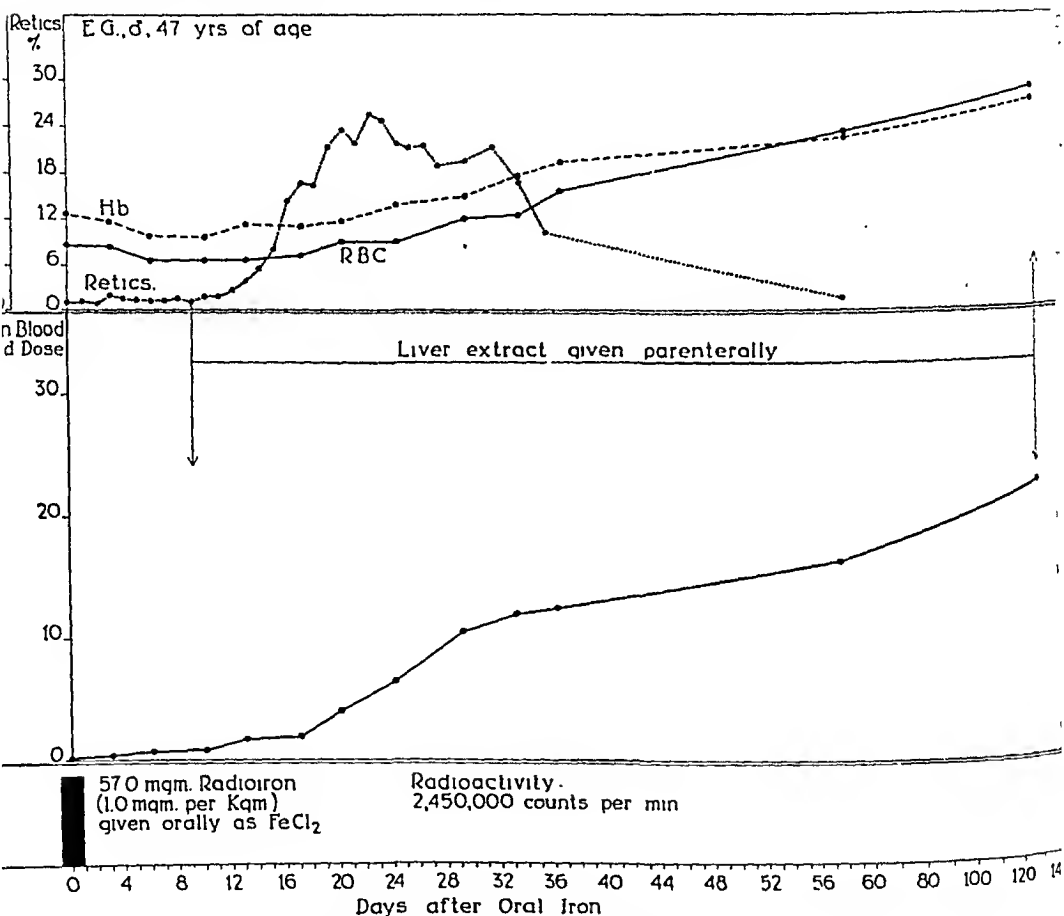


Fig. 10.—The appearance of radioactive iron in the blood of a patient with pernicious anemia after oral administration of ferrous chloride.

all of the classical clinical and hematologic manifestations of pernicious anemia in relapse, 1.0 mg. of radioactive iron per kilogram of body weight was given orally as ferrous chloride (Fig. 10). Only about 1 per cent of the dose appeared in the peripheral hemoglobin during the next nine days. This result was similar to that obtained by Ross and Chapin¹¹ and by Balfour and associates.⁹ According to interpretations made by these investigators, this result indicated that the patient had absorbed a very small percentage of the oral dose. On the ninth day, intramuscular injections of liver extract were begun. There was the

usual reticulocytosis, clinical improvement, and subsequent rise in both red blood cell and hemoglobin levels. Coincident with the rise in hemoglobin, additional amounts of radioactive iron made their appearance in the blood stream; eventually more than 20 per cent of the administered oral dose was found in the circulating hemoglobin. Similar results on four additional patients will be reported later. This observation clearly illustrates the caution which must be used in interpreting results of studies made with the radioactive iron technique. The patient just described was certainly not iron deficient; he had enough iron stored at the time the isotope was given to raise his hemoglobin from 5 to more than 13 Gm. per 100 cubic centimeters.

DISCUSSION

While iron utilization can be studied more satisfactorily with radioactive iron than with any other technique previously available, results are definitely influenced by the rate of red blood cell formation, the rate of red cell destruction, and possibly by other disturbances of hemoglobin metabolism. The importance of the first of these influences is relatively obvious. When erythrocytogenesis is depressed, new hemoglobin is being synthesized so slowly that only small amounts of the radioiron can be used. In hemolytic anemias, however, new hemoglobin is being formed at a rapid rate, and large quantities of iron are being used for this purpose. One would naturally expect that radioactive iron given to such subjects would be incorporated quantitatively and promptly into hemoglobin. Yet, only a portion of the administered dose can be recovered in circulating hemoglobin at any one time. There are two possible reasons for this unexpected result. The amount of untagged or nonradioactive iron available in the storage depots, and constantly being released from hemoglobin liberated from hemolyzed red blood cells, may be so great that the body barely samples the available radioactive isotope in order to have enough of the metal for hemoglobin formation. In the second place, new red cells containing radioiron live a rather short time because of the hemolytic process, and the concentration of radioactivity isn't built up. All of the administered radioiron might eventually be incorporated into hemoglobin, but only a portion is found in the peripheral blood at any one time.

One of the most interesting results was the prompt and almost complete utilization of the injected isotope by healthy adult men. From 70 to 90 per cent of the dose was used for hemoglobin synthesis within the first six to nine days. The newly administered iron, therefore, was selectively used in preference to body iron already stored. If the new iron had been intimately mixed with stored iron, the isotope would have been sampled only, and long periods of time would have been required before an appreciable quantity would have been used for hemoglobin formation. This suggests that iron recently added to the body (and, probably, that released each day from destroyed erythrocytes) is retained in some form which is more easily mobilized for current metabolic needs than is iron which has been stored for a longer period of time. If one represented stored iron as being present in a bottle, then iron added to the stores would both float on top as does cream in a bottle of milk and be removed

first when the bone marrow or other tissues needed it. As a result of his studies of ferritin, an iron-protein complex associated with iron storage, Granick¹⁹ postulates that there are three fractions of storage iron: *A*, monomolecularly dispersed iron compounds; *B*, colloidal iron compounds, namely, ferritin and noncrystallizable ferritin; and *C*, hemosiderin. Granick believes that *A* (ferrous and ferric compounds, probably in the form of their hydroxides) represents the metabolically active iron, which may be in equilibrium with *B* (ferritin). This impression is compatible with the suggestion just made. Similar ideas have recently been expressed by Ross⁵ and by Greenberg and Wintrobe.⁶ These workers postulate that there is a labile iron pool made up of recently absorbed iron and of iron recently liberated from destroyed red cells from which the body normally takes the amount needed each day. The concept is the same, but the term "labile iron pool" carries the unfortunate connotation that this amount may be held apart or separate from the remaining stores. It seems to us that the form of storage is simply one from which the iron is more readily mobilized.

This concept would explain many of the results obtained in this investigation. Normal dogs may use less of the injected radioiron for hemoglobin synthesis than do normal human subjects, because a greater amount of inert iron is available in the readily mobilizable form; the longer the isotope remains in tissues unutilized, the more inert iron becomes available from the hemoglobin liberated each day. This portion of the radioactive iron, therefore, becomes buried and is recalled only under conditions of great demand, as after extensive hemorrhage. The patient with acquired hemolytic anemia on whom a splenectomy was done recovered after the operation, and her hemoglobin level was restored to normal. Even with this rise, however, only small amounts of the isotope were extracted from her tissues for the hemoglobin regeneration, presumably because by this time the isotope had been mixed with so much inert iron equally available to the bone marrow that it was merely sampled rather than selectively used. On the other hand, one may assume that the patients with pernicious anemia used all of the isotope for hemoglobin synthesis following liver extract therapy because they were liberating hemoglobin from destroyed red cells at a rate too slow to "cover up" effectively the radioiron injected two weeks previously.

When comparison is made between the curves of appearance of radioactivity in the blood of subjects with hemolytic anemias and of patients with pernicious anemia before liver, a striking difference is noted. In the hemolytic anemias (Figs. 7 and 8), radioactivity increased very rapidly during the first few days and then rather promptly formed a plateau. In pernicious anemia, however, the rise was slow during the two weeks of observation prior to injections of liver extract (Fig. 6). This observation is interpreted as being additional presumptive evidence that hemolysis is not a prominent factor in the pathogenesis of the anemia in patients with pernicious anemia.

Finally, it should be emphasized that the radioactive iron technique when used on a living subject measures only the utilization of iron for hemoglobin synthesis; the amounts used for myohemoglobin and for formation of cellular enzymes cannot yet be followed.

SUMMARY AND CONCLUSIONS

1. The utilization of intravenously administered tracer doses of radioactive iron for hemoglobin synthesis has been studied in human subjects and in dogs, under normal conditions and in the presence of various types of anemia. It was found that:

(a) Utilization was prompt and complete in iron-deficient patients and dogs.

(b) Utilization by healthy adult men was somewhat slower but almost as complete. On the other hand, normal dogs used only 35 to 70 per cent of the injected isotope.

(c) Patients with hypoplastic anemia used less than 4 per cent of the tagged iron. In patients with pernicious anemia, utilization was likewise slow until after liver therapy was begun; it then was accelerated and became complete.

(d) In hemolytic anemias, the amounts of radioactive iron present in the peripheral blood at any one time varied widely from 10 to 90 per cent of the dose; rise in radioactivity was rapid during the first few days but usually stopped within a short time, so that the curves formed a plateau. However, in one dog which was made iron deficient before hemolysis was induced with phenylhydrazine, utilization was both prompt and complete during recovery from the hemolytic anemia.

(e) Utilization was slow and incomplete in patients with fevers caused either by infection or other associated disease. This result was obtained even when the subjects were also iron deficient.

2. To explain some of these results, it is postulated that iron recently made available for storage from absorption, parenteral administration, or from hemoglobin liberated from destroyed red blood cells is retained in a form which can be more readily mobilized for use than can iron which has been stored for longer periods of time. This recently stored iron seems to be used selectively for current metabolic needs.

3. Because injected radioactive iron is either not completely used for hemoglobin synthesis under a variety of conditions or does not all appear in the circulating hemoglobin at any one time, it is emphasized that the radioactive iron technique has limited value as a method of studying iron absorption from the gastrointestinal tract. An attempt has been made to define these limitations. The authors believe that the radioactive iron method for studying absorption is still the best available but desire to direct attention to its limitations so that results may not be interpreted erroneously. As an example, observations are presented to show that patients with untreated pernicious anemia may absorb considerable amounts of radioiron but that the isotope does not appear in the peripheral blood in large amounts until after erythrocyte maturation arrest has been relieved by administration of liver extract.

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SERUM LYSOLECITHIN IN RHEUMATOID ARTHRITIS, PREGNANCY, AND JAUNDICE AND IN NORMAL PERSONS

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THIS study was prompted by the observations of Bergenhem and Fahraeus¹ that lysolecithin was capable of causing spherocytosis and hemolysis of erythrocytes and that rapidly sedimenting blood after incubation shows normal erythrocyte sedimentation values. Since spherocytosis tends to prevent aggregation or rouleaux formation, it seemed desirable to determine to what extent the serum lysolecithin levels in rheumatoid arthritis were altered because there are increased sedimentation rates as well as mild anemic tendencies in this disease. Rouleaux formation of the erythrocytes is associated with increases in the rate of sedimentation.² Further, since pregnancy and jaundice are known to promote remission of symptoms in rheumatoid arthritis, the lysolecithin levels in these conditions were determined to see whether there was any similarity or difference in lysolecithin values.

A series of fifty serum lysolecithin determinations was made in patients with rheumatoid arthritis, pregnancy, jaundice, and in normal persons, as controls. At the same time determinations of erythrocyte sedimentation (Wintrobe method) and of packed-cell volume were made in an attempt to determine the possible relationship between the rate of erythrocyte sedimentation and serum lysolecithin content.

Method for Lysolecithin Determination.—The method used was a modification of that described by Singer³ and was as follows: 10 c.c. of serum were treated with 100 c.c. of 95 per cent ethyl alcohol and filtered immediately; the filtrate was evaporated under a stream of air; the residue was then taken up in 5 c.c. of 95 per cent ethyl alcohol and diluted with 30 c.c. of ethyl ether and centrifuged for ten minutes at 20,000 r.p.m.; the supernatant fluid was decanted off and the precipitate dried in a vacuum jar. Then the precipitate was dissolved in 0.8 c.c. of physiologic saline solution, and dilutions were made in geometric series. A 2 per cent standard erythrocyte suspension was prepared using the blood of the same normal individual for all determinations. The standard cells were washed three times in physiologic saline solution and then suspended in saline solution. This cell suspension was drawn up to the 0.5 mark on standard leucocyte pipettes and diluted with the lysolecithin solutions to the 11 mark. The pipettes were then incubated at 37° C. for one hour, and cell counts were made in the usual manner in the hemocytometer. The percentage of hemolysis was determined by comparing the cell counts in each of the serial dilutions to those of the controls using physiologic saline solution instead of the lysolecithin solutions.

Summary of Results.—In all cases the erythrocytes appeared to become more or less spheroid in the presence of the lysolecithin as compared with the normal controls in physiologic saline solution (0.85 per cent NaCl). The amounts of hemolysis in ten patients with rheumatoid arthritis, ten icteric patients, twenty

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TABLE I. PER CENT HEMOLYSIS IN RHEUMATOID ARTHRITIS

PATIENT	C.E.S.R.* (MM.)	DILUTIONS (PER CENT)							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
G. N.	46	93	40	0	0	0	0	0	0
P. A.	42	97	25	0	0	0	0	0	0
M. C. N.	22	95	68	16	0	0	0	0	0
G. N.	45	45	35	0	0	0	0	0	0
B. X.	3	86	80	35	0	0	0	0	0
B. E.	30	100	87	86	70	30	16	0	0
R. S.	35	100	100	0	0	0	0	0	0
H. S.	28	100	56	48	26	0	0	0	0
S. L.	12	65	30	10	0	0	0	0	0
B. T.	37	48	39	30	24	20	18	0	0
Average		83	56	23	12	5	3	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

TABLE II. PER CENT HEMOLYSIS IN ICTERUS

PATIENT	DIAGNOSIS	C.E.S.R.* (MM.)	I. I.†	DILUTIONS (PER CENT)							
				1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
S. W.	Hepatitis, acute	24	40	49	30	5	2	0	0	0	0
C. N.	Hemolytic anemia	6	20	66	35	20	8	0	0	0	0
S. S.	Hemolytic anemia	4	30	32	26	15	0	0	0	0	0
J. N.	Hepatitis, chronic	8	20	25	21	0	0	0	0	0	0
B. M.	Hepatitis, chronic	21	50	90	77	36	0	0	0	0	0
W. T.	Hepatitis, chronic	34	30	100	92	73	32	0	0	0	0
S. O.	Hepatitis, chronic	10	50	79	48	28	25	20	0	0	0
W. D.	Hepatitis, chronic	29	50	0	0	0	0	0	0	0	0
M. N.	Hepatitis, acute	14	80	67	36	36	36	26	26	0	0
H. N.	Hepatitis, chronic	34	190	97	88	0	0	0	0	0	0
Average				61	47	21	10	5	3	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

†I.I., icteric index.

TABLE III. PER CENT HEMOLYSIS IN PREGNANCY

PATIENT	MONTH PREGNANT	C.E.S.R.* (MM.)	DILUTIONS (PER CENT)								
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
M. N.	5	18	33	20	12	0	0	0	0	0	0
W. N.	7	22	28	23	14	14	14	17	17	0	0
B. N.	6	10	23	16	16	16	23	11	0	0	0
G. A.	6	24	77	59	4	0	0	0	0	0	0
R. Z.	7	35	86	48	50	66	40	0	0	0	0
H. S.	4	22	35	23	0	0	0	0	0	0	0
J. R.	7	25	37	42	22	22	18	15	0	0	0
M. L.	4	14	80	55	60	37	36	18	18	0	0
K. O.	7½	19	52	53	25	14	8	2	0	0	0
M. I.	7½	21	60	0	0	0	0	0	0	0	0
V. E.	4	12	60	50	12	0	0	0	0	0	0
W. N.	6	25	15	0	0	0	0	0	0	0	0
H. X.	6	10	38	0	0	0	0	0	0	0	0
S. T.	5	24	0	0	0	0	0	0	0	0	0
S. T.	4	29	42	15	0	0	0	0	0	0	0
K. Y.	5	34	11	16	16	0	0	0	0	0	0
F. N.	6	22	11	0	0	0	0	0	0	0	0
H. N.	6	38	30	30	0	0	0	0	0	0	0
G. H.	4	36	48	30	0	0	0	0	0	0	0
K. Y.	5	14	71	45	11	6	0	0	0	0	0
Average			84	53	24	17	14	6	4	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

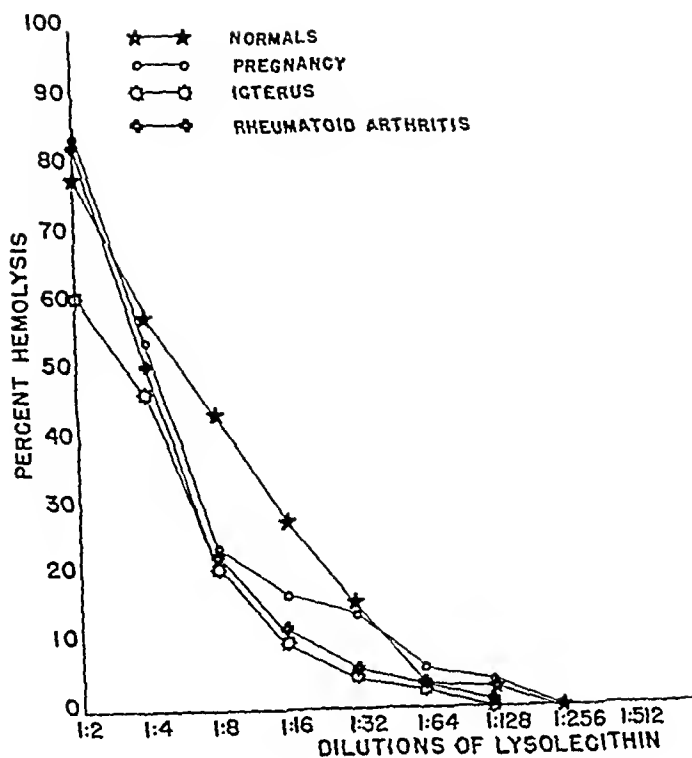


Fig. 1.

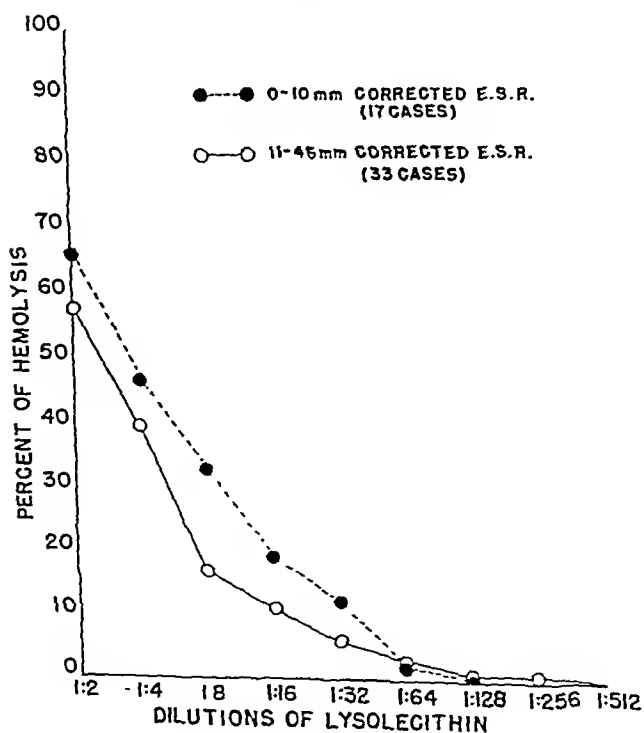


Fig. 2.

TABLE IV. PER CENT HEMOLYSIS IN NORMAL PERSONS

PERSON	C.E.S.R.* (MM.)	DILUTIONS (PER CENT)							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
K. L.	2	100	100	98	60	44	30	30	0
V. E.	0	75	67	56	28	0	0	0	0
C. G.	6	36	20	0	0	0	0	0	0
E. T.	2	97	74	51	43	34	0	0	0
D. M.	3	88	77	74	71	60	0	0	0
N. R.	6	100	17	10	0	0	0	0	0
C. E.	7	95	95	70	58	20	0	0	0
A. I.	4	87	38	36	20	0	0	0	0
H. N.	3	52	43	30	0	0	0	0	0
G. N.	0	50	44	15	0	0	0	0	0
Average		78	58	44	28	16	3	3	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

pregnant patients, and ten normal persons are given in Tables I to IV, respectively. It is seen that in each table there was considerable variation from one patient to another as manifested by the percentage of hemolysis in the serial dilutions of lysolecithin. In other words, there was not a close adherence to a definite hemolysis curve for any of the conditions studied. Fig. 1 shows the average curves for each of the conditions studied.

The results are also grouped according to rate of erythrocyte sedimentation in Fig. 2 which shows the average curves for the seventeen cases with sedimentation rates of 0 to 10 mm. per hour and the thirty-three cases with sedimentation rates from 11 to 46 mm. per hour. It is seen that the characteristic average curves for each condition did not vary appreciably from one another. Furthermore, the lysolecithin content as manifested by hemolysis was not significantly different in those subjects with sedimentation rates below 10 mm. per hour and in those whose rates were over 10 mm. per hour. This was especially apparent when it is remembered that this method of determination is subject to about a 10 per cent error in measuring the percentage of hemolysis.

CONCLUSIONS

1. The average serum lysolecithin values, as measured by the amount of hemolysis of standard human erythrocytes, were found not to vary appreciably in rheumatoid arthritis, pregnancy, jaundice, and in normal persons as controls, but the individual variations were marked in these conditions.

2. There was a close correlation between the average lysolecithin values of persons with erythrocyte sedimentation rates below 10 mm. per hour and with those between 10 and 46 mm. per hour; therefore, the amount of lysolecithin did not appear to be related to the rate of erythrocyte sedimentation.

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THE RETAINED MEDIAN LETHAL DOSE, PERCENTAGE RETENTION, AND RESPIRATORY RESPONSE IN UNANESTHETIZED, INTACT RABBITS AND IN UNANESTHETIZED, TRACHEOTOMIZED DOGS AND RABBITS EXPOSED TO PHOSGENE BY THE DOSIMETRIC METHOD

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THE apparent species difference in susceptibility to phosgene previously reported on the basis of $L(Ct)_{50}$ data¹ was not resolved by determining the retained median lethal dose per kilogram of body weight in normal, unanesthetized dogs,² goats,³ and monkeys.⁴ However, only two of the factors, percentage retention and respiratory intake during exposure, which contribute to the variability of the $L(Ct)_{50}$ were eliminated by measuring the toxicity in terms of retained dose per kilogram of body weight.

Since the characteristic toxic effects of phosgene are exerted only on direct contact with the lower respiratory tract,⁵ the proportion of retained agent which is absorbed by the respiratory epithelium above this locus of action may affect the apparent resistance of the animal to phosgene poisoning. Consequently, anatomic and physiologic dissimilarities in the upper respiratory tracts of different species may account, in part, for species variations in susceptibility.

To evaluate the influence of absorption in the upper respiratory tract on the apparent sensitivity to phosgene, the retained median lethal dose was determined in unanesthetized dogs and rabbits in which the upper respiratory tract was by-passed by tracheotomy under local (procaine) anesthesia. Ease of handling and availability of dogs and rabbits led to the use of these animals as species representing high and low resistance¹ to phosgene, respectively. To check the marked susceptibility of rabbits previously reported on the basis of $L(Ct)_{50}$ data,¹ the retained median lethal dose was determined in a series of thirty-two intact, unanesthetized rabbits.

The dosimetric technique permitted the simultaneous determination of the percentage retention and the respiratory response during exposure.⁶ Because some of these data suggested that depth of respiration influenced the percentage retention and the toxicity of phosgene, two groups of tracheotomized rabbits were studied, one breathing deeply with artificial respiration and the other breathing more shallowly without mechanical aid.

METHODS

All experimental exposures to phosgene were conducted at chamber concentrations of approximately 2.0 mg. per liter which, due to the dead space in the apparatus,² correspond to the following actual mean contact concentrations:

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† Ct = concentration \times time.

1.48 mg. per liter for the thirty-five tracheotomized dogs; 1.25 mg. per liter for the thirty-two deep-breathing, intact rabbits; 1.52 mg. per liter for the thirty-two deep-breathing, tracheotomized rabbits; and 1.30 mg. per liter for the forty-three shallow-breathing, tracheotomized rabbits. The apparatus, procedures, and methods used for the dogs and rabbits in these experiments were similar to those previously employed in the dosimetric exposure of normal dogs² and monkeys,⁴ respectively, except that for the tracheotomized animals no facepiece was required, since the distal end of each glass tracheal cannula consisted of the male half of a standard tapered joint for connection with the valve inlet of the apparatus.

The tracheotomies were performed under local anesthesia (1 c.c. of a 2 per cent solution of procaine hydrochloride for rabbits and 2 c.c. for dogs). After the exposure to phosgene, the cannula was removed carefully, and sulfathiazole crystals were applied in the cleansed wound which was left open for drainage. No deaths were observed in fifteen control rabbits and ten control dogs similarly cannulated and treated but not exposed to phosgene. The mean temperature and relative humidity during the exposures of the tracheotomized rabbits were 84.6° F. and 68.6 per cent, respectively; these corresponding data during exposures of the intact rabbits were 78.1° F. and 41.9 per cent, respectively, while those during the exposures of the dogs were 84.0° F. and 69.0 per cent, respectively.

In the intact normal rabbits, connection to the smaller glass valves and absorber was made by means of a vinylite nosepiece sealed to the male portion of a ground-glass joint. Great care was necessary to avoid obstructing the external nares. To test for possible leakage of the facepiece, a series of fifteen rabbits was prepared, as for dosimetric exposure, and permitted to inhale air from one spirometer and to exhale into another through the facepiece, valves, and absorber. In contrast to dogs, goats, and monkeys,²⁻⁴ it was found that in rabbits, despite the most careful efforts to obtain an airtight seal, when the facepiece was applied to the snout with Ace elastic adhesive bandage, considerable leakage occurred (mean leakage, 56.4 per cent; standard deviation = ± 15.2 per cent; and standard error = 4.0 per cent). Therefore, the LD_{50} for rabbits, as determined with the leaking facepiece, was calculated and corrected by applying a factor of 1.77, based on the mean leakage observed in these tests. The standard error of the corrected LD_{50} was calculated as the sum of the standard error of the uncorrected LD_{50} plus the standard error of the mean leakage observed in the tests. It is recognized that the uncorrected standard error of the median lethal dose already includes the variation caused by leakage. However, to be conservative, the two standard errors were combined. Because of the leakage, the authors do not recommend that this method for dosimetrically exposing nontracheotomized rabbits be used.

As a result of the marked reduction in tidal air during exposure of the intact rabbits, the small pressure changes transmitted to the valve system resulted in small fluttering movements of the flexible vinylite sheeting and produced very slight, slow respiratory deflections of the spirometer. To shorten the exposure times and to minimize rebreathing, artificial respiration was employed to increase tidal air and respiratory minute volumes.

RESULTS

The calculations and statistical treatment of these data were the same as described in a previous paper.² The complete experimental data for each animal are given in Tables I to IV.

Respiratory Response.—Respiratory inhibition in the normal, untracheotomized rabbits was so intense that, as previously stated, artificial respiration was employed to accelerate the exposures to phosgene. Therefore, no quantitative data were obtained on the spontaneous respiratory changes in these rabbits during exposure to phosgene.

None of the tracheotomized animals manifested any alteration of respiration during exposure, in contrast to the intact dogs,² goats,³ monkeys,⁴ and rabbits, which almost uniformly exhibited marked inhibition of respiration on exposure to similar concentrations of phosgene. The large, mean respiratory minute volume of 819 c.c. per kilogram per minute (Table V) and the relatively short exposures of the shallow-breathing, tracheotomized rabbits (Table III) indicate the absence of such inhibition in these animals.

The data in Table V permit a comparison between the average respiratory minute volume per kilogram during exposure of the normal and the tracheotomized dogs, which were 294 and 419 c.c. per kilogram per minute, respectively. The value (3.0) obtained by Student's *t* test reveals that the differences between these means is statistically significant (that is, $t \leq 3$). Because dogs pant in response to increased environmental temperature and because there was a temperature difference of 7.3° F., comparison between the respiratory exchange in the two groups must be made cautiously. However, panting, nontracheotomized dogs also exhibit unequivocal respiratory inhibition exposure to phosgene. The absence of any respiratory inhibition in the tracheotomized dogs and the presence of the marked inhibition in the nontracheotomized animals clearly indicates that the inhibition results from the irritant action of phosgene on the upper respiratory tract.

Probably another consequence of the much higher temperatures during the exposures of the tracheotomized dogs is the fact that the average tidal air (Table VI) during exposure of this group is less than one-half that observed for the normal dogs.² The *t* value (9.8) reveals that this difference is highly significant.

The average tidal air of the shallow-breathing, tracheotomized rabbits, respiring without mechanical aid, was less than half that of the tracheotomized rabbits subjected to deep artificial respiration during exposure (Tables II and III). The *t* value (6.3) indicates that this difference is highly significant. The average respiratory minute volumes for the shallow-breathing and the deep-breathing tracheotomized rabbits were 819 and 408 c.c. per kilogram per minute, respectively. The *t* value (7.1) also indicates the marked significance of the difference between these two means. The influence of respiratory amplitude and rate of flow on toxicity and percentage retention of phosgene will be discussed.

Median Lethal Dose (LD₅₀).—The retained median lethal dose and the statistical range were calculated for each series by the method of Bliss.⁷ After preliminary grouping of the animals to establish the provisional dosage-mortality regression line, each animal was considered a separate experiment. Agreement

TABLE I. PERTINENT DATA FOR THIRTY-TWO UNANESTHETIZED, INTACT, NORMAL RABBITS SUBJECTED TO DEEP ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 Mg. PER LITER

PHOSGENE EXPOSURE										PHOSGENE RETENTION				RESPIRATION		SURVIVAL (HR.)
SEX	WEIGHT (KG.)	VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./ LITER)	TIME (MIN.)	CT† CORRECTED (γ-MIN./ LITER)	TOTAL (MG.)	DOSE RETAINED		PER CENT RETENTION		CORREC- TION FACTOR	ACTUAL	NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)		
							(MG.)	(MG./ KG.)								
F	2.61	0.16	1.71	-	-	0.27	0.01+	0.01	-	-	3.1	-	-	-	Survived	
F	3.07	0.16	1.72	-	-	0.28	0.03	0.01	-	-	10.9	-	-	-	Survived	
F	2.79	0.18	1.71	-	-	0.31	0.03	0.02	-	-	16.2	-	-	-	Survived	
M	2.87	0.19	1.71	-	-	0.33	0.07	0.02	-	-	20.7	-	-	-	Survived	
F	2.50	0.19	1.72	-	-	0.33	0.07	0.03	-	-	21.2	-	-	-	Survived	
F	3.64	0.22	1.71	-	-	0.38	0.19	0.05	-	-	50.4	-	-	-	Survived	
F	3.18	0.33	1.60	-	-	0.53	0.17	0.03	-	-	32.4	-	-	-	Survived	
F	2.79	0.23	1.73	-	-	0.40	0.18	0.06	-	-	41.6	-	-	-	Survived	
F	3.95	0.38	1.81	-	-	0.68	0.29	0.07	-	-	42.0	-	-	-	Survived	
F	2.61	0.20	1.82	-	-	0.36	0.17	0.07	-	-	47.8	-	-	-	48	
F	3.35	0.31	1.79	-	-	0.56	0.27	0.08	-	-	48.9	-	-	-	48	
F	3.41	0.44	1.79	-	-	0.79	0.28	0.08	-	-	35.9	-	-	-	Survived	
M	2.76	0.24	1.69	-	-	0.41	0.23	0.08	-	-	55.3	-	-	-	Survived	
F	3.64	0.28	1.69	-	-	0.41	0.23	0.09	-	-	56.9	-	-	-	Survived	
M	2.87	0.40	1.60	-	-	0.18	0.32	0.09	-	-	65.6	-	-	-	Survived	
F	2.99	0.64	1.70	-	-	0.64	0.25	0.09	-	-	39.0	-	-	-	48	
F	2.76	0.32	1.78	-	-	1.09	0.26	0.09	-	-	23.5	-	-	-	Survived	
F	3.52	0.45	1.69	-	-	0.57	0.28	0.10	-	-	49.1	-	-	-	Survived	
F	2.44	0.48	1.75	-	-	0.76	0.38	0.11	-	-	49.6	-	-	-	Survived	
F	3.12	0.60	1.75	-	-	0.85	0.39	0.16	-	-	46.3	-	-	-	Survived	
F	2.86	0.63	1.77	-	-	1.05	0.50	0.16	-	-	47.6	-	-	-	Ca. 72	
F	2.58	0.96	1.77	-	-	1.12	0.44	0.16	-	-	39.7	-	-	-	Ca. 21	
F	2.30	0.86	1.70	-	-	1.63	0.46	0.18	-	-	28.3	-	-	-	Survived	
F	2.81	0.96	1.73	-	-	1.48	0.57	0.25	-	-	38.2	-	-	-	Ca. 18	
F	2.84	1.11	1.70	-	-	1.63	0.75	0.27	-	-	45.8	-	-	-	Ca. 21	
F	3.35	1.02	1.72	-	-	1.91	0.78	0.28	-	-	11.0	-	-	-	Ca. 18	
F	2.98	1.00	1.80	-	-	1.83	0.99	0.30	-	-	51.2	-	-	-	Ca. 18	
F	2.47	0.86	1.72	-	-	1.80	1.01	0.34	-	-	56.2	-	-	-	Ca. 20	
F	3.38	1.37	1.82	-	-	1.48	0.86	0.35	-	-	58.1	-	-	-	Ca. 18	
F	2.47	1.01	1.79	-	-	2.49	1.32	0.39	-	-	52.8	-	-	-	Ca. 5	
F	2.58	1.13	1.72	-	-	1.81	1.00	0.40	-	-	55.0	-	-	-	Ca. 18	
						1.91	1.16	0.45	-	-	59.8	-	-	-	Ca. 18	

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct

*Chamber concentration × Correction factor = Mean contact concentration.
 †Ct × Correction factor = Corrected Ct.

TABLE II. PERTINENT DATA FOR THIRTY-TWO UNANESTHETIZED, TRACHEOTOMIZED RABBITS SUBJECTED TO DEEP ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 Mg. PER LITER

RABBITS TRACHEOTOMIZED		PHOSGENE EXPOSURE				PHOSGENE RETENTION				RESPIRATION		SURVIVAL (HR.)
		VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./LITER)	TIME (MIN.)	Ct† CORRECTED (γ-MIN./LITER)	DOSE RETAINED		PER CENT RETENTION		NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	
						(MG.)	(MG./KG.)	OBSERVED	CORRECTION FACTOR			
NO.	WEIGHT (KG.)											
1	2.27	0.416	1.965	0.98	423	0.270	0.119	32.9	77.0	25	16.65	Survived Ca. 16
2	1.73	0.172	2.065			0.211	0.122	59.5				Survived
3	2.10	0.236	2.015	0.70	1,080	0.302	0.144	63.3	76.5	15	15.75	Survived
4	2.84	0.240	2.200	0.41	726	0.408	0.144	77.4	80.5	11	22.00	Survived
5	1.37	0.188	1.740	0.36	492	0.209	0.153	61.4	78.5	10	18.80	Survived
6	2.95	0.276	2.590	1.03	2,110	0.696	0.155	65.6	81.3	12	23.00	Survived
7	2.84	0.268	2.190	0.50	982	0.587	0.158	76.3	82.5	12	22.00	Ca. 16
8	2.73	0.260	2.240	0.30	552	0.437	0.160	75.0	80.5	9	26.00	Survived
9	2.36	0.468	1.900	0.51	771	0.889	0.163	43.8	79.6	23	20.35	Ca. 16
10	2.73	0.316	2.200	0.20	376	0.695	0.166	65.1	84.7	10	32.00	Survived
11	2.61	0.948	1.715	0.76	908	0.474	0.182	27.0	69.7	103	9.20	Survived
12	1.82	0.340	1.745	0.25	372	0.594	0.197	61.0	85.3	10	34.00	Survived
13	1.88	0.608	1.850	0.70	954	0.426	0.227	38.5	73.6	48	12.66	Survived
14	1.59	0.316	1.745	0.35	515	0.372	0.233	67.2	84.5	10	31.60	Ca. 16
15	2.04	0.470	1.810	0.23	336	0.330	0.255	38.8	80.8	21	22.39	Survived
16	1.00	0.320	2.055	1.60	2,514	0.658	0.261	75.4	76.5	20	16.00	Survived
17	2.61	0.492	2.250	0.53	960	0.690	0.261	62.3	80.5	22	22.00	Ca. 16
18	2.16	0.472	1.800	0.50	761	0.568	0.263	66.9	84.5	15	31.40	Survived
19	2.55	0.532	1.830	0.91		0.678	0.266	70.0				Survived
20	3.01	0.552	2.250	1.19	2,089	0.880	0.293	71.0	78.0	30	18.00	Ca. 16
21	2.61	0.520	2.250	0.41	775	0.783	0.296	66.8	84.0	18	29.00	Ca. 16
22	1.79	0.400	2.060	1.00	1,738	0.824	0.307	66.7	94.4	13	30.78	Ca. 16
23	1.88	0.600	1.675	0.34	437	0.624	0.332	59.0	76.8	37	16.22	Ca. 16
24	2.47	0.812	1.875	0.36	494	0.894	0.362	58.5	73.2	67	12.12	Ca. 16
25	1.93	0.596	1.915	0.44	750	0.729	0.378	64.0	80.0	49	12.18	Ca. 24
26	1.85	0.576	1.965	2.40	3,580	0.775	0.420	69.6	76.0	38	15.17	Ca. 16
27	1.79	0.508	2.060	1.00	1,694	0.822	0.459	78.6	82.1	20	25.04	Survived
28	1.67	0.528	2.050	1.70	1,851	0.770	0.461	70.8	81.8	25	21.15	Survived
29	1.67	0.536	2.060	1.10	1,804	0.774	0.464	70.0	79.6	26	20.60	Ca. 16
30	2.22	0.712	2.050	1.50	1,414	1.078	0.485	73.8	78.5	38	18.75	Ca. 16
31	2.39	0.888	2.080	1.40	2,413	1.362	0.570	73.3	82.5	34	26.10	Ca. 16
32	1.88	1.088	1.745	1.00	1,495	1.345	0.715	70.8	85.7	31	35.11	Ca. 16

*Chamber concentration × Correction factor = Mean contact concentration.

†Ct × Correction factor = Corrected Ct.

TABLE III. PERTINENT DATA FOR FORTY-THREE UNANESTHETIZED, TRACHEOTOMIZED RABBITS BREATHING WITHOUT ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 MG. PER LITER

RABBITS			PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		SURVIVAL (HR.)
TRACHEOTOMIZED			VOLUME (LITERS)	CHAMBER CONCEN- TRATION* (MG./ LITER)	TIME (MIN.)	CT† COR- RECTED (% MIN./ LITER)	TOTAL (MG.)	DOSE RETAINED		PER CENT RETENTION			NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.O.)	
NO.	WEIGHT (KG.)							(MG.)	(MG./ KG.)	OBSERVED	CORREC- TION FACTOR	ACTUAL			
1	2.27		0.272	2.01	0.11		0.517	0.118	0.061	27.1				Survived	
2	2.59		0.240	2.02	0.07		0.483	0.178	0.069	36.7				Survived	
3	2.50		0.304	2.01	0.19		0.611	0.206	0.082	33.7				Survived	
4	1.47		0.224	2.00	0.11		0.448	0.126	0.086	28.1				Survived	
5	2.50		0.300	2.01	0.08		0.603	0.323	0.129	33.1				Survived	
6	1.47		0.320	2.01	0.23		0.613	0.206	0.110	32.1				Survived	
7	2.20		1.264	1.90	0.75	9.55	2.402	0.372	0.169	15.5	67.0	23.1	171	Survived	
8	2.05		0.764	1.70	0.54	6.25	1.296	0.376	0.181	29.1	68.3	42.6	91	Survived	
9	1.93		0.912	1.73	0.46	5.36	1.579	0.371	0.192	23.5	67.1	31.9	122	Survived	
10	2.95		0.932	1.96	0.37		1.827	0.581	0.198	32.0				Survived	
11	2.73		0.880	1.96	0.11		1.720	0.550	0.201	32.0				Survived	
12	3.07		1.612	1.76	0.70	8.52	2.836	0.626	0.201	22.1	69.2	31.9	181	Ca. 16	
13	2.13		1.568	1.70	0.73	8.89	2.658	0.558	0.202	21.0	71.8	29.2	141	Survived	
14	2.95		0.924	2.02	0.43		1.867	0.797	0.270	12.7				Survived	
15	2.03		1.496	1.68	1.14	12.19	2.511	0.561	0.274	22.3	65.2	34.2	242	Survived	
16	2.02		1.468	1.74	0.87	1.019	2.527	0.557	0.276	22.1	67.5	32.6	191	Survived	
17	2.24		1.276	1.90	0.37	7.21	2.421	0.668	0.278	27.6	66.9	41.2	177	Ca. 16	
18	2.33		1.676	1.71	0.71	8.71	2.863	0.673	0.289	23.5	68.8	34.2	197	Ca. 16	

19	1.82	0.380	1.97	0.48	918	1.142	0.537	0.290	46.2	69.3	36.4	172	8.82	Survived
20	2.13	1.516	1.74	0.68	707	2.516	0.610	0.300	25.3	67.4	36.2	167	7.19	Survived
21	1.73	1.352	1.72	0.61	897	2.155	0.595	0.303	25.9	66.7	38.8	224	7.02	Survived
22	2.13	1.576	1.68	0.67	730	2.616	0.656	0.322	25.9	78.2	73.7	36	18.11	Ca. 90
23	2.05	0.652	1.99	0.40	1,087	1.293	0.715	0.363	57.6	66.8	37.4	234	7.11	Ca. 16
24	1.99	1.664	1.85	0.88	1,087	3.080	0.769	0.386	25.0	67.7	62.0	114	7.68	Survived
25	1.62	0.876	1.73	0.86	1,016	1.530	0.642	0.396	12.0	68.8	30.0	253	8.48	Ca. 16
26	2.27	2.114	1.97	1.43	1,939	1.223	0.903	0.398	21.1	73.4	52.8	114	12.39	Ca. 16
27	2.15	1.412	1.90	0.64	803	2.653	1.001	0.433	39.6	69.4	34.1	214	8.90	Ca. 85
28	1.97	2.172	1.95	1.21	1,634	1.223	0.998	0.440	24.6	65.2	65.5	132	6.18	Survived
29	1.34	0.940	1.76	1.11	1,270	1.615	0.895	0.452	34.8	69.6	43.3	227	8.69	Survived
30	2.36	1.972	1.90	0.95	1,253	3.737	1.132	0.480	30.3	65.6	63.0	200	6.38	Ca. 16
31	1.96	1.276	1.87	1.41	1,607	2.383	0.986	0.503	11.4	73.0	66.7	108	11.96	Ca. 16
32	2.24	1.392	1.90	1.21	1,679	2.453	1.195	0.533	18.7	69.8	54.3	200	9.18	Ca. 16
33	2.36	1.836	1.84	1.14	1,463	3.378	1.278	0.542	37.9	66.3	53.3	295	6.84	Ca. 16
34	2.30	2.524	1.79	0.80	947	3.692	1.272	0.553	35.3	57.7	50.1	31	12.82	Ca. 16
35	2.02	1.328	1.90	2.33	3,882	2.508	1.123	0.556	14.2	80.2	47.2	188	11.13	Ca. 16
36	2.50	2.092	1.82	1.31	1,907	3.790	1.435	0.574	37.9	69.0	69.5	133	8.63	Survived
37	1.73	1.148	1.80	1.27	1,657	2.166	1.041	0.602	18.1	67.1	60.6	232	7.27	Ca. 16
38	1.03	1.688	1.79	0.75	838	3.014	1.228	0.636	10.7	68.0	18.3	280	8.27	Ca. 16
39	2.11	2.316	1.94	1.67	2,923	4.491	1.494	0.656	33.2	67.2	80.2	172	7.33	Ca. 20
40	1.93	1.264	1.80	0.92	1,168	2.490	1.287	0.666	53.9	67.2	25.4	137	7.05	Ca. 16
41	1.73	0.956	1.90	1.09	1,384	1.818	0.308	0.755	16.9	66.8	99.2	498	2.54	Ca. 16
42	1.90	1.464	1.93	2.54	2,850	2.824	1.567	0.825	55.5	57.6	99.2	222	11.56	Ca. 16
43	2.75	2.568	1.98	1.20	1,990	5.083	2.283	0.830	45.4	83.7	54.8	222		Ca. 16

*Chamber concentration \ Correction factor = Mean contact concentration.

†Ct x Correction factor = Corrected Ct.

TABLE IV. PERTINENT DATA FOR THIRTY-FIVE UNANESTHETIZED, TRACHEOTOMIZED DOGS EXPOSED BY DOSIMETRIC METHOD TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 Mo. PER LITER

DOGS TRACHEOTOMIZED		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION	
		VOLUME (LITERS)	CHAMBER CONCEN- TRATION* (MG./ LITER)	TIME (MIN.)	CT† COR- RECTED (γ-MIN./ LITER)	TOTAL (MG.)	DOSE RETAINED		PER CENT RETENTION		NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	
							(MG.)	(MG./ KG.)	OBSERVED	CORREC- TION FACTOR			ACTUAL
SEX	WEIGHT (KG.)												SURVIVAL (HR.)
M	10.45	10.628	1.96	3.13	4,250	20.83	6.43	0.615	30.9	69.2	41.6	37.2	Survived
F	9.55	10.565	1.99	2.49	3,430	20.98	5.93	0.621	28.3	69.2	40.8	37.3	Survived
M	8.18	10.496	2.06	3.76	5,382	21.62	5.72	0.699	26.4	69.2	38.0	38.7	Survived
F	6.36	8.664	1.89	2.63	3,395	16.31	4.49	0.706	27.5	68.3	40.2	31.6	Ca. 87
M	14.13	13.160	1.90	3.59	5,061	25.00	10.25	0.727	41.0	74.2	55.3	56.2	Survived
F	10.23	10.560	1.93	2.65	5,380	20.38	8.03	0.783	39.4	69.3	56.8	37.8	Survived
M	9.10	9.440	1.90	1.30	1,706	17.94	7.91	0.791	41.3	69.1	61.0	36.9	Survived
M	10.91	10.684	2.11	3.87	5,608	22.56	8.91	0.816	39.5	68.7	57.3	33.5	Survived
F	10.90	16.390	1.98	8.42	11,300	32.37	9.12	0.836	28.2	67.8	41.6	33.1	Ca. 22
F	10.91	11.176	1.98	4.12	5,840	22.07	8.27	0.819	42.0	71.6	58.7	45.1	Survived
F	10.45	10.432	1.91	2.91	3,926	19.92	9.92	0.893	45.3	70.1	64.7	41.1	48
F	17.50	16.612	1.91	4.42	6,321	31.73	15.43	0.882	48.6	74.9	61.9	59.3	Ca. 90
F	5.91	8.092	1.94	3.52	4,864	13.70	5.53	0.939	35.3	71.2	49.8	41.0	Survived
M	10.68	13.860	2.05	2.93	5,938	28.42	10.17	0.932	35.8	73.9	48.5	51.8	Survived
M	6.36	9.036	1.96	1.93	2,658	17.81	6.16	0.993	31.6	70.2	49.3	40.8	Survived
M	9.80	8.660	2.03	3.94	5,980	17.75	10.20	0.985	57.5	76.0	75.7	65.2	Survived
F	11.82	13.452	1.95	1.30	1,880	26.23	11.83	1.001	45.2	74.2	60.9	56.0	Survived
M	7.27	7.332	1.91	2.27	3,088	14.00	7.30	1.001	52.2	71.2	73.3	44.1	Survived
F	11.40	14.960	2.05	2.89	4,332	30.67	11.47	1.005	37.1	73.1	51.2	51.5	Survived
M	13.60	13.740	2.00	3.50	5,250	27.18	13.68	1.005	49.8	75.0	66.5	60.3	Survived
M	15.91	17.092	2.04	2.61	4,242	34.87	16.37	1.029	47.0	78.8	59.7	80.9	27
F	7.27	7.356	1.96	0.97	1,393	14.41	7.31	1.033	52.1	73.2	71.3	51.8	Survived
F	12.10	17.310	2.03	4.60	6,450	35.49	12.61	1.045	35.6	68.1	52.0	31.9	Survived
M	13.60	16.950	1.95	8.21	11,955	33.08	14.38	1.037	43.3	71.1	58.5	57.3	Survived
M	13.40	11.710	2.00	4.11	6,205	23.12	13.87	1.070	59.2	76.5	77.1	67.3	Ca. 40
F	13.60	25.278	2.00	4.60		50.56	15.16	1.116	30.5				Survived
F	6.82	7.496	2.06	1.82	2,700	15.11	7.89	1.157	51.1	72.0	71.0	47.0	Survived
F	5.45	6.408	1.92	2.86	3,650	12.28	4.63	1.217	51.0	66.6	81.0	29.9	Ca. 36
M	6.36	9.236	1.93	1.84	2,549	17.83	7.93	1.216	41.5	71.8	62.0	46.6	Ca. 24
F	5.68	6.272	2.00	3.10	4,546	12.55	7.10	1.302	59.0	73.3	80.5	52.3	Survived
F	7.27	7.872	1.98	4.18	6,374	15.55	10.65	1.165	68.5	77.0	88.9	69.7	Ca. 21
F	10.91	17.688	1.92	5.43	7,540	33.81	16.09	1.173	47.5	72.3	65.7	48.1	Ca. 16
F	6.36	9.768	2.03	4.20	6,700	19.86	10.16	1.397	51.2	78.5	73.2	53.7	Ca. 16
M	10.91	16.704	1.97	8.17	13,170	32.83	19.63	1.709	50.9	81.8	73.2	60.8	Ca. 16
M	9.30	15.040	2.04	2.05	3,145	30.61	20.86	2.242	69.1	75.2	90.5	69.6	Ca. 24

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct.

TABLE V. AVERAGE RESPIRATORY MINUTE VOLUME PER KILOGRAM BODY WEIGHT IN NORMAL AND TRACHEOTOMIZED DOGS AND IN SHALLOW- AND DEEP-BREATHING, TRACHEOTOMIZED RABBITS DURING EXPOSURE TO PHOSGENE CONCENTRATIONS OF 1.30 TO 1.52 PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	RESPIRATORY MINUTE VOLUME PER KILOGRAM DURING EXPOSURE		
		MEAN (C.C./MIN./KG.)	STANDARD DEVIATION (C.C./MIN./KG.)	STANDARD ERROR (C.C./MIN./KG.)
Normal dogs	30	294	118	21
Tracheotomized dogs	35	419	217	37
Shallow-breathing tracheotomized rabbits	43	819	266	41
Deep-breathing tracheotomized rabbits	32	408	234	41

TABLE VI. AVERAGE TIDAL AIR VOLUME PER KILOGRAM BODY WEIGHT IN NORMAL AND TRACHEOTOMIZED DOGS AND IN DEEP- AND SHALLOW-BREATHING RABBITS DURING EXPOSURE TO PHOSGENE CONCENTRATIONS OF 1.30 TO 1.52 MG. PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	AVERAGE TIDAL AIR VOLUME PER KILOGRAM BODY WEIGHT		
		MEAN (C.C./KG.)	STANDARD DEVIATION (C.C./KG.)	STANDARD ERROR (C.C./KG.)
Normal dogs	30	11.56	2.97	0.56
Tracheotomized dogs	35	5.18	1.92	0.33
Tracheotomized rabbits subjected to deep, artificial respiration	32	10.4	4.14	0.76
Tracheotomized rabbits breathing without artificial respiration	43	4.5	3.16	0.55

between the provisional and the calculated dosage-mortality regression lines was tested by calculating chi square (X^2).⁷

The median lethal dose of phosgene for the unanesthetized, tracheotomized dogs was 1.20 mg. per kilogram, with a range of 0.82 to 1.76 mg. per kilogram for $P = 0.01$. The LD_{50} for the unanesthetized, tracheotomized rabbits was 0.33 mg. per kilogram, with a range of 0.24 to 0.45 mg. per kilogram for $P = 0.01$, for those which were not subjected to artificial respiration during exposure, and 0.25 mg. per kilogram, with a range of 0.15 to 0.42 mg. per kilogram for $P = 0.01$, for those subjected to deep artificial respiration. The LD_{50} for the intact rabbits subjected to artificial respiration was 0.20 mg. per kilogram, with a range of 0.16 to 0.24 mg. per kilogram (Table VII).

In Table VIII are listed the t values obtained when comparisons are made between the LD_{50} 's of phosgene for the various series of animals reported in this paper. These t values and the one obtained by comparing the tracheotomized dogs with the normal dogs previously reported² indicate that: (1) eliminating the upper respiratory tract by tracheotomy does not produce a significant change in the LD_{50} for dogs or rabbits; (2) there is a highly significant difference between the LD_{50} 's for tracheotomized dogs and tracheotomized rabbits; and (3) although increasing the depth and decreasing the minute volume of respiration in tracheotomized rabbits tends to decrease the LD_{50} , the decrease is not statistically significant.

Percentage Retention of Inhaled Phosgene.—Calculation of the percentage of inhaled material retained after dosimetric exposure is dependent on the aver-

TABLE VII. LD₅₀'S FOR UNANESTHETIZED, TRACHEOTOMIZED AND INTACT, NORMAL DOGS AND RABBITS EXPOSED TO MEAN PHOSGENE CONCENTRATIONS OF APPROXIMATELY 1.5 MG. PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	LD ₅₀ (MG./KG.)	RANGE OF LD ₅₀ FOR P = 0.01 (MG./KG.)	STANDARD ERROR (MG./KG.)
Normal rabbits subjected to deep, artificial respiration	32	0.20	0.16 to 0.24	0.015
Trach. rabbits subjected to deep artificial respiration	32	0.25	0.15 to 0.42	0.052
Trach. rabbits breathing without artificial respiration	43	0.33	0.24 to 0.45	0.042
Normal dogs ²	30	1.31	1.09 to 1.59	0.097
Tracheotomized dogs	35	1.20	0.82 to 1.76	0.183

TABLE VIII. *t** VALUES OBTAINED WHEN LD₅₀'S OF PHOSGENE FOR TRACHEOTOMIZED, UNANESTHETIZED DOGS AND RABBITS AND FOR NORMAL RABBITS ARE COMPARED BY STUDENT'S *t* TEST

SPECIES	TRACHEOTOMIZED DOGS	DEEP-BREATHING TRACHEOTOMIZED RABBITS	SHALLOW-BREATHING TRACHEOTOMIZED RABBITS	DEEP-BREATHING NORMAL RABBITS
Tracheotomized dogs	—	4.97	4.65	5.29
Deep-breathing tracheotomized rabbits	4.97	—	1.11	0.7
Shallow-breathing tracheotomized rabbits	4.65	1.11	—	2.05
Deep-breathing normal rabbits	5.29	0.7	2.05	—

$$* t = \frac{m_1 - m_2}{\sqrt{(E_1)^2 + (E_2)^2}} \quad (m = \text{Median lethal dose.})$$

$$\quad \quad \quad (E = \text{Standard error.})$$

age tidal air during exposure.² In the intact rabbits with marked inhibition of respiration, many individual breaths could not be identified on the spirometer records, despite artificial respiration. Therefore, the average tidal air could not be estimated accurately, and the percentage retention could not be calculated.

The mean percentage of inhaled phosgene retained by the tracheotomized rabbits subjected to deep artificial respiration was 78.3 per cent, with a standard deviation of 15.8 per cent and a standard error of 2.9 per cent. The mean percentage retention for the shallow-breathing, tracheotomized rabbits was 48.1 per cent, with a standard deviation of 21.0 per cent and a standard error of 3.7 per cent. The *t* value (6.1) confirms the marked significance of this difference.

The mean percentage retention of phosgene in the tracheotomized dogs was 61.7 per cent, with a standard deviation of 13.8 per cent and a standard error of 2.4 per cent. The *t* value (4.6) establishes the statistical significance of the difference between this mean and that of 74 per cent previously reported² for normal dogs.

DISCUSSION

Boyd and Perry⁸ investigated the influence of the nasobuccal pharyngeal filter on the toxicity of phosgene. They reported that, in rabbits and cats but not in dogs, by-passing the upper respiratory tract by tracheotomy before exposure to 0.30 to 0.45 mg. per liter of phosgene for thirty minutes produced markedly greater mortality than was observed in intact animals of the respective species exposed to the same concentration of phosgene. The difference be-

tween the several species was attributed to the fact that little phosgene is removed and thus rendered innocuous by the nasal mucosa of the dog, which is a mouth-breather in warm weather, in contrast to the nose-breathing rabbits and cat.

However, the influence of respiratory volume on the dose of phosgene acquired during conventional chamber exposures cannot be ignored. Inasmuch as there is no decrease in the respiratory intake of tracheotomized animals during exposure to moderate concentrations of phosgene, such animals must inhale more agent during a given period than do intact animals in which contact of phosgene with the nasal mucosa produces marked respiratory inhibition. Since this respiratory inhibition is of variable duration in different species, the species differences which Boyd and Perry have observed in the effect of tracheotomy on mortality following Ct exposure to phosgene for thirty minutes may be due to the fact that in the presence of phosgene, intact rabbits can maintain markedly reduced respiration for long periods, whereas intact dogs do not inhibit respiration significantly for more than a minute or two.^{2, 9} Consequently, the course of the influence of the upper respiratory tract on the toxicity of phosgene cannot be evaluated by chamber exposures.

The results of our experiments indicate that the susceptibility of dogs and rabbits to the toxic action of phosgene is not significantly affected by the elimination of the upper respiratory tract by tracheotomy. Since the actual dose of phosgene retained was measured, uncontrolled differences in respiratory intake during exposure could not affect survival. Apparently, the proportion of inhaled phosgene which is absorbed by the upper respiratory tract at the concentrations studied is too small and inconstant to produce statistically significant changes in the median lethal dose, particularly when the variability (statistical range) is great, due either to heterogeneity or to the small size of the sample of the experimental population, or to other factors previously discussed.² In this connection it is interesting that, by direct measurement, Cameron, Gaddum, and Short¹⁰ observed that at nominal concentrations of 40, 100, and 500 mg. per m³ "phosgene passes through the nose without marked loss, although 25 per cent difference in phosgene concentration of air entering and leaving the nose is not uncommon." Later, they¹⁰ found that at higher concentrations, equivalent to those to which our dogs and rabbits were exposed, only 2 to 4 per cent of phosgene was removed by the nasal mucosa of monkeys.

In the tracheotomized dogs the depth of respiration was significantly less and the respiratory minute volume greater than in the normal group. If the toxicity of phosgene increases as respiration becomes deeper and slower, this might have masked a significant change in the LD₅₀ after tracheotomy. To explore this possibility further, the LD₅₀ was determined in a group of tracheotomized rabbits in which, by means of artificial respiration, the depth of respiration was increased to more than twice that of the first group of tracheotomized rabbits, and the respiratory minute volume was correspondingly decreased. As previously noted, however, although the LD₅₀ for the shallow-breathing rabbits was 40 per cent greater than that for the deep-breathing rabbits, the statistical spread for the two groups was too great to permit the demon-

stration of significance in this difference. A larger series with more uniform control of depth and minute volume of respiration might clarify this point further.

A highly significant difference between the percentage of phosgene retained by the deep-breathing and by the shallow-breathing tracheotomized rabbits was demonstrated. An increase in the percentage of inhaled gas retained as the tidal air volume is increased and the rate of airflow is decreased would be expected, since, with increased depth of respiration, a greater proportion of the inhaled vapors comes into direct contact with pulmonary respiratory epithelium and the slower air flows increase time of contact. In the earlier experiments on normal dogs,² goats,³ and monkeys,⁴ in which the depth of respiration was not controlled, this relationship was obscured. However, the decreased percentage of phosgene retained by the tracheotomized dogs as compared with the more deeply breathing, normal dogs probably is attributable, in part, to the shallower, more rapid respiration of the former group.

The failure to explain the reported species differences in susceptibility to phosgene by eliminating the upper respiratory tract indicates that as yet undefined factors must be responsible. These data suggest that one such factor may be depth of respiration.

SUMMARY

1. The median lethal dose, the per cent of inhaled agent retained, and the respiratory changes during dosimetric exposure to phosgene were determined, after by-passing the upper respiratory tract by tracheotomy, in thirty-five unanesthetized dogs, in thirty-two unanesthetized rabbits which were subjected to deep, artificial respiration during exposure, and in forty-three unanesthetized rabbits which breathed more shallowly and rapidly without artificial respiration.

2. In addition, thirty-two normal, unanesthetized rabbits were subjected to artificial respiration during dosimetric exposure to a mean phosgene concentration of approximately 1.25 mg. per liter. The median lethal dose for this group was 0.20 mg. per kilogram (standard error = 0.015 mg. per kilogram). Because of leakage of the facepiece, the dosimetric technique described is not recommended for use in untracheotomized rabbits.

3. The median lethal dose for the dogs which were exposed to an average phosgene concentration of 1.48 mg. per liter was 1.20 mg. per kilogram (standard error = 0.18 mg. per kilogram). This is not significantly different from the LD_{50} (1.31 mg. per kilogram) previously reported for untracheotomized dogs exposed at approximately the same concentration.

4. The median lethal dose for unanesthetized, tracheotomized rabbits was 0.25 mg. per kilogram (standard error = 0.052 mg. per kilogram) for a group of thirty-two, which was subjected to deep, artificial respiration during exposure to a mean phosgene concentration of 1.52 mg. per liter, and 0.33 mg. per kilogram (standard error = 0.042 mg. per kilogram) for the group of forty-three, which breathed more shallowly and rapidly without artificial respiration during exposure to a mean phosgene concentration of 1.30 mg. per liter. The decrease in the LD_{50} produced by the increase in depth and decrease in velocity of respira-

tory flow is, however, not statistically significant. Moreover, as in dogs, the LD_{50} 's in tracheotomized and in intact rabbits are not significantly different. Therefore, since the susceptibility of both dogs and rabbits to phosgene apparently is not significantly affected by by-passing the upper respiratory tract during exposure, previously reported differences in the resistance of these two species to phosgene poisoning are not explained by this procedure.

5. The mean percentages of inhaled phosgene retained were as follows: 61.7 per cent (standard deviation = ± 13.8 per cent; standard error = ± 2.4 per cent) for the tracheotomized dogs; 78.3 per cent (standard deviation = ± 15.8 per cent; standard error = ± 2.9 per cent) for the deep-breathing, tracheotomized rabbits; and 48.1 per cent (standard deviation = ± 21.0 per cent; standard error = ± 3.7 per cent) for the shallow-breathing, tracheotomized rabbits. No data on percentage retention could be obtained in the intact rabbits. The mean percentage retentions for the tracheotomized dogs and the shallow-breathing, tracheotomized rabbits are significantly less than that of 74 per cent previously observed for a group of untracheotomized dogs and that for the deep-breathing rabbits, respectively. Since in the tracheotomized dogs and in the shallow-breathing, tracheotomized rabbits the mean tidal air was much smaller and the mean minute volume was greater than in the normal dogs and in the deep-breathing rabbits, respectively, these differences demonstrate the influence of depth and minute volume of respiration on the percentage of the agent retained after inhalation.

6. In contrast to intact dogs, goats, monkeys, and rabbits, none of the tracheotomized rabbits or dogs, in which contact with inhaled vapors was limited to the respiratory tract below the larynx, exhibited any signs of respiratory inhibition or irritation during exposure to phosgene.

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CHROMOGENIC REACTIONS TO THE HALOGENATION OF PREGNANCY URINE

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GERMAN patent No. 680223 advances the claim for a pregnancy test requiring only a few minutes to perform and displaying a high degree of accuracy. The reaction is dependent on the production of a rose color when a solution of iodine is added to pregnancy urine, the color being characterized by its solubility in isoamyl alcohol. Schales and Schales¹ have made an effort to identify the substance responsible for this phenomenon, but we know of no other reports on the procedure.

TECHNIQUE

The iodine reagent was prepared by adding 1.5 Gm. of iodine and 2.5 Gm. potassium iodide to 100 c.c. of water.¹ Using glacial acetic acid and/or NaOH, the urine is adjusted to a pH of 6.0 to nitrazine paper. Five cubic centimeters are placed in each of two test tubes and the tubes immersed for thirty seconds in a beaker of boiling water. As soon as the tubes are removed, 0.2 c.c. of the iodine reagent is added to one and 0.4 c.c. to the other specimen. The solutions are agitated gently for five seconds, and 2 c.c. of isoamyl alcohol is added to each. After shaking vigorously for a moment the tubes are allowed to stand until the alcohol has stratified above the urine. A rose or pink color in the alcohol layer of either tube is read as positive. All other color changes or no color are read as negative.

Results.—The test was carried out 300 times. In eight cases (2.7 per cent) the reading was doubtful. Of the remaining 292 patients, 203 were pregnant, and the result was positive in 159 (78.3 per cent). In the eighty-nine non-pregnant patients, negative reports were obtained in sixty-nine (77.5 per cent). This same material is rearranged in Table I to provide more information from the clinician's viewpoint. From this it can be seen that given a positive report there is about an 11 per cent chance of error, while the negative report carries almost a 40 per cent error.

Comment.—The claims made for the test cannot, on the basis of this work, be substantiated, and this degree of inaccuracy rules out the procedure as an aid in the diagnosis of pregnancy. The routine checking of such a claim, however, while a necessary task, does not completely eliminate the reaction from further consideration. In the first place, it represents a chromogenic phenomenon which occurs more frequently in the pregnant than in the nonpregnant woman, and from this point of view it merits consideration. Furthermore, a certain amount of interest is attached to the test since it is the third time that halogenation of the urine has been advanced as a color reaction for pregnancy.

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TABLE I

<i>Positive Tests (179)</i>	
Patient pregnant	88.8%
Patient not pregnant	11.2%
<i>Negative Tests (113)</i>	
Patient pregnant	39.0%
Patient not pregnant	61.0%

Friedrich² advocated a test based on the addition of HCl to urine and the development of a dark red-brown color, while the histidine test, which has had a more extensive trial,³ rests on the color developed on the bromization of pregnancy urine. In neither the iodine nor the hydrochloric acid reactions has the responsible chromogenic substance been determined, and the question arises as to the identity or parallelism between these procedures.

The Color Change.—There is a diminution in the amount of color developed as the pH of the urine tested shifts either way from the optimum point of six. As noted by Schales and Schales,¹ charcoal will adsorb the chromogen, but other adsorption and ion-exchange media (aluminum earth, zeolites, and synthetic resins) did not remove it. Preliminary treatment of the urine with permuit, in fact, intensifies the final color somewhat.

The chromogen itself is not heat labile, but leaving the tubes immersed in the boiling water bath for two minutes or longer elevates the temperature to a point where the color will not develop. On the other hand, the reaction will not occur when the iodine reagent is added to urine at room temperature. The solubility in isoamyl alcohol refers only to the final pigment and does not apply to the substance responsible for the reaction. Thus, preliminary extraction of the urine with this alcohol, either at room temperature or after gentle heating, does not alter the color developed.

The relationship between the results of the test and the duration and nature of the pregnancy is not consistent. The distribution of false negatives in the various trimesters is equal, and the presence or absence of albuminuria or lactosuria does not influence the results. Consecutive daily tests during the immediate puerperium were run on ten patients who had had positive reactions at the time of delivery. Six of these turned negative between the fourth and the sixth post-partum day, while the remainder continued to excrete the chromogen until the time of discharge. One patient, however, after two negative reports on the fourth and the fifth post-partum days again excreted the chromogen on the sixth day, and in the course of selecting these cases, a patient was discovered who had a negative test at delivery but turned positive on the first post-partum day. These results stress the inconsistency of the test with relation to the status of the pregnancy.

RELATIONSHIP TO OTHER HALOGENATION PROCEDURES

Hydrochloric Acid Test.—A few drops of 25 per cent HCl are added to 1 e.e. of urine having a specific gravity of 1.015. After heating a few minutes a dark-brown or a deep red-brown color is considered positive, while a light brown or other color is reported negative. With this reaction Friedrich reported 100 per cent accuracy.²

In a series of sixty tests performed by us, thirty on pregnant and thirty on nonpregnant urines, the Friedrich test produced 20 per cent false positive and 50 per cent false negative results. Coincidental performance of the iodine test on these same urine specimens revealed no identity or parallelism in the reaction of the two chromogenic substances. The combined results obtained from these simultaneous procedures (that is, both tests positive, both negative, or a conflicting result) could not be correlated with the clinical diagnoses and did not improve the accuracy of the laboratory reports.

While their behavior with various adsorption media differs, the most striking dissimilarity between the two chromogens lies in their reaction to heat. The rose color cannot be developed when the temperature of the urine is above 90° C., whereas the color developed on adding HCl is accentuated by prolongation of the heating.

Histidine.—That most nontoxemic pregnant women excrete histidine has long been known. That the phenomenon is not consistent enough to provide a pregnancy test is also acknowledged. The recent excellent work of Page⁴ has shown that the reaction is apparently due to a lowering of the renal threshold for histidine during pregnancy.

Histidine gives a violet color on bromization, which has formed the basis for most of the histidine tests described. A solution of histidine hydrochloride when mixed with the iodine reagent does not, however, yield the characteristic rose color, and the behavior of the rose pigment toward various adsorption media differs from that of histidine. The rose-color reaction under discussion apparently is not dependent on nor related to the presence or absence of histidine.

In the course of comparing the two procedures, however, it was noted that whenever both tests were positive on the same sample of urine the patient was invariably pregnant. The coincidence of a positive rose-color reaction and a positive histidine reaction carried an almost negligible diagnostic error. The question was raised, accordingly, as to the frequency with which, in a series of cases, the two tests coincided with positive reactions.

The bromization technique of Page³ is too sensitive for qualitative purposes, and with it almost all urines are positive. The procedure was therefore modified to "desensitize" it by treating the urine initially with Lloyd's reagent and omitting the addition of sodium urate. One gram of Lloyd's reagent was used for each 10 c.c. of urine and filtered out after five minutes. Three-tenths of a gram of charcoal was used in the step removing urinary pigments, and, since both adsorption media remove some histidine, this technique yielded positive results only in the face of a high concentration of histidine.

Results.—The rose-color reaction and the histidine test were carried out on 253 samples of urine. Both tests were positive in ninety-one cases (36 per cent) and negative in 73 (29 per cent), while the results of the two procedures disagreed in the remaining eighty-nine cases (35 per cent). Of the ninety-one patients with whom both procedures were positive, ninety (98.8 per cent) were pregnant and one was nonpregnant. However, when both tests were negative there was a 22 per cent error attendant on the diagnosis of nonpregnant, and in the face of conflicting reports from the two tests no diagnosis could be made

at all. Of the 253 patients, 176 were pregnant, and the "double positive" occurred in only ninety (51 per cent).

Comment.—In so far as the hydrochloric acid reaction goes, it is doubtful that there is a single chromogenic substance responsible for the color change. Actually the procedure is apparently a modified acid hydrolysis, and while it is true that the hormone content of a urine specimen can effect the color developed on acid hydrolysis, it is also obvious that the initial pigment content of the urine as well as its nonhormonal salts will have a decided influence.

In contrast to this, the development of the rose color seems to rest on a definite chromogenic substance. While the exact nature of the substance is undetermined, it would appear to be neither hormonal nor specifically related to pregnancy. The degree of diagnostic accuracy for the simultaneous occurrence of positive readings in the iodine and bromine reactions is remarkable, although the fundamental basis for such a finding remains obscure. Aside from presenting an avenue for investigation, however, the combined procedures have a dubious value, and it is questionable that there is any immediate clinical application for the double test. Although the two determinations can be carried out in twenty minutes, two out of every three performed yielded a report from which no conclusion could be drawn. In one case out of every three (in this series) a result was obtained which provided over 98 per cent accuracy in the diagnosis of pregnancy.

CONCLUSION

1. A rose color, soluble in isoamyl alcohol, is produced when an iodine solution is added to some specimens of urine. While this reaction occurs more frequently in the pregnant than the nonpregnant woman, the claim that it provides a suitable diagnostic test for pregnancy cannot be substantiated on the basis of the studies herein reported.

2. The color reaction which does take place is not related to the HCl reaction proposed by Friedrich as a pregnancy test nor to the histidine content as determined by bromization.

3. The observation was made in the course of this study that a coincidental positive rose-color and positive histidine test was associated with pregnancy in 98.8 per cent of the cases. In contrast to this high degree of specificity, however, the sensitivity is only 50 per cent, and the excretion of the rose chromogen bears no consistent relationship to either the duration or the nature of the pregnancy.

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SOME OBSERVATIONS ON SEVERE DIABETIC KETOSIS TREATED WITH GLUCOSE AND INSULIN

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AUGUSTA, GA.

THE present report is concerned with some observations on acetone bodies and glucose in blood and urine, which were made on a group of patients with diabetic coma, or threatened coma, who were treated with glucose and insulin. The results seem to bear on some of the unsettled problems in this field.

METHODS

Acetone and total acetone bodies were determined by the method of Greenberg and Lester.¹ The quantities of reagents and sample solutions were increased fivefold, so that a volume of 10 c.c. of carbon tetrachloride extract was obtained for reading in the Evelyn colorimeter. The digestion for total acetone bodies was carried out under a reflux condenser with standard taper ground-glass connections.

Qualitative clinical tests of the acetone bodies in the urine were made by: (1) the nitroprusside technique of Rabinowitch²; and (2) a modified Gerhardt technique: boil about 10 c.c. of urine for two minutes; if a cloud develops, add a drop of strong HCl, and if it is still turbid, filter. Transfer 4 c.c. of the boiled urine to one tube and 4 c.c. of the fresh urine to a similar tube. To each add 2 c.c. of a solution containing 50 Gm. FeCl₃ and 12 c.c. concentrated HCl per liter. As ordinarily carried out, the ferric chloride test is difficult to assay in the presence of salicylate, especially if a little protein is also present. With the technique described here, relatively small concentrations of diacetic acid may be detected by a difference of color intensity in the two tubes.

Quantitative determinations of glucose were made, at first by an adaptation of the technique of Hoffman,³ subsequently by the Nelson-Somogyi⁴ colorimetric procedure.

The Ketosis in Relation to the Diagnosis of Diabetic Coma.—Two examples of diabetic coma are provided by Cases 3 and 4 in Table I, with the urine giving a negative reaction with ferric chloride and only a mild reaction with nitroprusside. The concentrations of acetone in blood and urine are similar in these cases, as in all of the others; however, the total acetone bodies of the urine in these two cases are at levels lower than those found in the blood. These observations are, therefore, in confirmation of others^{5, 6} which indicate that too much reliance should not be placed on tests for diacetic acid in the urine in the diagnosis of diabetic coma.

On the admission specimens the free acetone content of the blood ranges from 20 to 32 per cent of the total acetone. These figures are of the same order as those reported by Marriott.⁷ Since the free acetone of the blood always amounts to a substantial proportion of the total acetone and since the acetone of the alveolar air bears a mathematical relation to the blood acetone, a test for acetone in alveolar air should give an index of the extent of the ketosis. A simple clinical method for the estimation of acetone in alveolar air has been described previously.⁸

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TABLE I. SHOWING CHANGES IN BLOOD AND URINE OF PATIENTS WITH SEVERE DIABETIC ACIDOSIS DURING TREATMENT WITH GLUCOSE AND INSULIN

	HOURS AFTER BLOOD NO. 1	BLOOD			URINE				
		QUANTITATIVE MG. %			QUANTITATIVE MG. %		QUALITATIVE		
		ACE- TONE	TOTAL ACE- TONE BODIES	GLU- COSE	ACE- TONE	TOTAL ACE- TONE BODIES	FeCl ₃	NITRO- PRUS- SIDE	SUGAR
Case 1									
Blood and urine No. 1	0	23.7	90.6	889	22.2	319.2	+++	++++	++++
50 units insulin S.C.	0								
1 liter 5% glucose-saline I.V.	2								
Blood and urine No. 2	4	23.2	68.2	668			++	++++	++++
50 units insulin S.C.	4								
1 liter 5% glucose-saline I.V.	6								
Blood and urine No. 3	12.5	9.2	32.4	363	10.8	57.8	+	+++	++++
Case 2									
Blood and urine No. 1	0	5.8	28.1	358	5.1	63.3	+	+++	++++
50 units insulin S.C.	0								
1 liter 5% glucose-saline I.V.	1								
Blood and urine No. 2	10.5	2.5	14.4	122	3.1	15.4	Neg.	+	+++
Case 3									
Blood No. 1	0	25.6	114	680	26.2	63	Neg.	++	++++
50 units insulin S.C.	0								
plus 50 units I.V.	1								
1 liter 5% glucose-saline I.V.	2								
Blood No. 2 and urine No. 2	3	24.4	67	615	22.4	71	Neg.	++	++++
Case 4									
Blood No. 1	0	29.4	91	478	29.7	86	Neg.	++	++++
150 units insulin S.C.	0								
3 liters 5% glucose-saline I.V.	6								
Blood No. 2 and urine No. 2	12	6.4	13	230	6.9	14	Neg.	Trace	++
Case 5									
Blood and urine No. 1	0	33.2	111.0	641	37.6	741.0	++++	++++	++++
50 units insulin I.V. plus 150 units S.C.	0								
2 liters 5% glucose-saline I.V.	3.5								
Blood and urine No. 2	4.5	31.9	59.0	361	37.4	165.0	++	++++	++++
50 units insulin S.C.									
50 Gm. glucose plus orange juice orally	6								
Blood and urine No. 3	10.5	24.1	36.4	75	24.2	44.8	Neg.	++	+

(Table I cont'd on following page)

TABLE I.—CONT'D

	HOURS AFTER BLOOD NO. 1	BLOOD			URINE				
		QUANTITATIVE MG. %			QUANTITATIVE MG. %		QUALITATIVE		
		ACE- TONE	TOTAL ACE- TONE BODIES	GLU- COSE	ACE- TONE	TOTAL ACE- TONE BODIES	FeCl ₃	NITRO- PRUS- SIDE	SUGAR
Case 6									
Blood and urine No. 1	0	32.2	102.0	930			++++	++++	++++
150 units insulin I.V.	0								
plus 200 units S.C.									
500 c.c. plasma plus 500									
c.c. saline I.V.									
3 liters 5% glucose-	10								
saline I.V.									
Blood and urine No. 2	16	13.8	39.5	428			Neg.	++	++++
Case 7									
Blood and urine No. 1	0	21.5	94.0	507	27.8	436.5	+++	++++	++++
100 units insulin S.C.	0								
2 liters 5% glucose-	6								
saline I.V.									
Blood and urine No. 2	14	17.8	54.7	64	19.0	77.0	+	+++	+

Saline was administered subcutaneously to each of these patients during the first few hours of treatment. The insulin employed was "regular" soluble insulin. The figures for acetone bodies represent acetone equivalents.

On the Use of Glucose in the Treatment of Coma.—This has been a very controversial subject. That administration of glucose is not necessary in the treatment of coma, providing the laboratory facilities are such that blood chemistry can be obtained as desired at any time of the day or night, is certain from the reports of Joslin⁹ and Root.¹⁰ Among the objections to the use of glucose which have been suggested are the following: (1) Glucose neutralizes insulin. It is true that glucose will prevent insulin hypoglycemia, but according to Drury and Palmer,¹¹ the activity of insulin is greater at high blood sugar levels. (2) Intravenous administration of glucose solutions will aggravate diuresis and dehydration. This is true for strong glucose solutions in distilled water but not for 5 per cent glucose in saline. (3) Glucose administration interferes with the use of blood sugar as a basis of control. This is a valid objection. However, the objection would be a more important one if the level of blood sugar could be taken as an accurate index of the extent to which the metabolic disturbance is corrected. That this is not always true is shown by the results in Cases 2, 5, and 7. In each of these, the level of the blood sugar had returned to the normal range when the last blood was taken, but the ketosis in each instance was far from complete correction. Accordingly, tests which indicate the extent of the ketosis are suggested as an alternative basis of control. Determination of the plasma bicarbonate may be substituted for the determination of total acetone bodies. Although lowering of bicarbonate is not due entirely to ketone acids, as pointed out by Martin and Wiek,¹² the lowering of the bicarbonate level is considered by Joslin and co-workers⁵ as a reliable index of the diabetic acidosis, and improvement in the plasma bicarbonate has been suggested as a basis of control by Almy, Swift, and Tolstoi.¹³ Except for cases with suppressed ex-

cretion, the intensity of the ketosis is indicated in a satisfactory manner by the tests for diacetic acid in the urine. As illustrated by the results in the table, the test with ferric chloride becomes negative, first, and then fading of the test with nitroprusside follows.

The following are among the advantages associated with the use of glucose: (1) The administration of glucose lessens the chance of hypoglycemia. This is particularly important in the elderly patient with diabetes with coronary disease. It is well known¹⁴ that such patients are prone to attacks of angina with hypoglycemia or even with a sudden drop of blood sugar from a chronic high level to the normal range. In Cases 2, 5, and 7, the return to normal would have been considered too rapid for elderly subjects; more glucose would have been administered. (2) The patient in diabetic coma is in need of carbohydrate.¹⁵ His stores of glycogen are to a certain extent depleted, and intravenous administration of glucose is the most feasible method for carbohydrate replacement and for providing nourishment until he is able to take food by mouth. According to the calculations of Joslin,⁹ who minimizes the need, the total carbohydrate of the patient in coma may be depleted to an average extent of about 30 Gm., and Joslin concedes the need for carbohydrate to cover half the caloric requirement for the first day, which would amount to about 175 Gm. for a man weighing 70 kilograms. In other words, such a patient should have at least 200 Gm. of glucose in the first twenty-four hours. The patients who were the subjects of this study received from 100 to 200 Gm. within the first few hours. Certainly this was not too much. In each instance, by the time the last blood sample was taken, the administered glucose had been disposed of and part of the patient's blood sugar as well. In Cases 5 and 7, a state of mild hypoglycemia had developed.

In view of the observed results, it is suggested: that the patient in diabetic coma should receive glucose in quantities equivalent to at least half the caloric requirements; that administration of insulin might be controlled by tests which indicate the intensity of the ketosis; and that determinations of sugar in blood and urine be carried out in order to safeguard against hypoglycemia.

SUMMARY

Observations have been made on changes in the concentrations of sugar and acetone bodies in blood and urine during the treatment of diabetic coma with insulin and glucose. From the observed results, suggestions have been made concerning the appropriate quantities of glucose which should be administered and concerning appropriate tests for diagnosis and control.

Acknowledgment is made for clinical assistance to Dr. J. M. Martin, Dr. J. L. Mulherin, Dr. Phyllis Johnson, Dr. J. L. Lokey, and Dr. J. W. Hurst.

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THE EFFECT OF GLYCEROPHOSPHATE ON BLOOD COAGULATION AND ON BLOOD CLOT RETRACTION

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THE anticoagulant effect of soluble glycerophosphates has been previously mentioned.¹ Since the solubility of calcium glycerophosphate, like the citrate, has a negative temperature coefficient, it seemed likely that calcium glycerophosphate would be poorly ionized and that glycerophosphate might inhibit coagulation by calcium ion depressant effect. This proved to be the case. Glycerophosphated plasma is readily coagulated by the addition of calcium chloride, sulfate, or dibasic phosphate, and whole glycerophosphated blood showed an unusual delay in spontaneous hemolysis on storage. Glycerophosphate found a limited application as an anticoagulant for blood specimens that had to be transported for long distances under unfavorable conditions prior to special serologic examination; however, since a final concentration of 2 per cent glycerophosphate in the plasma was found necessary for complete coagulation inhibition, the utility of this anticoagulant was limited by expense.

When less than 2 per cent glycerophosphate was added to freshly drawn blood, coagulation was delayed; however, after the advent of the latter, clot retraction appeared to be accelerated. Because there are clinical conditions in which clot retraction is deficient, this effect was further studied.

EXPERIMENTAL

Eastman's sodium glycerophosphate (52 per cent alpha) and Merek's beta preparation were used. Both gave essentially the same results. After dissolving the substance in water, the solutions were brought to pH 7.4 by the addition, dropwise, of concentrated HCl.

Blood was collected directly from the arm vein of human subjects by the insertion of a 3 inch, 20 gauge needle and allowed to flow directly down the glycerophosphated, solution-moistened wall of a graduated centrifuge tube containing measured quantities of 5, 10, or 20 per cent sodium glycerophosphate solution. During collection the centrifuge tube was continuously agitated by tapping the bottom to facilitate admixture with the anticoagulant solution, which, because of its high viscosity and specific gravity, otherwise tends to stratify at the bottom.

Coagulation time was measured by the Lee-White method² and clot retraction time by that of Macfarlane,³ with the necessary modification that these determinations were made within the centrifuge tube into which the blood collection had been made.

RESULTS

While a final concentration of 2 per cent or over of sodium glycerophosphate was found necessary to prevent coagulation indefinitely in most samples tested, in some, as little as 0.04 per cent would cause sensible inhibition. In other samples, 1 per cent glycerophosphate would delay coagulation for several hours. More constant, however, is the effect of subminimal anticoagulant quantities of sodium glycerophosphate on clot retraction time which is definitely diminished, as shown by representative experiments in Table I. Not amenable to quantitative representation is the fact that clots in glycerophosphated blood appear to shrink more rapidly and to attain a smaller final volume than in the control blood samples.

TABLE I. EFFECT OF SODIUM GLYCEROPHOSPHATE ON COAGULATION AND CLOT RETRACTION TIME

SAMPLE	GLYCEROPHOSPHATE (PER CENT)	CLOTTING TIME (MINUTES)	CLOT RETRACTION TIME (MINUTES)
Be	0.00	3.5	70
	0.25	5.0	40
	0.50	6.0	25
Myk	0.00	3.5	55
	0.10	4.5	35
	0.20	6.0	30
	0.50	9.0	20
Gi	0.00	5.0	80
	0.50	7.0	45
	1.00	8.5	30
	1.50	14.0	40
	2.00	No clot	

It might be mentioned that in the blood of a patient with terminal acute myeloid leucosis, with extensive purpura and a thrombocyte count of 28,000, the addition of 0.25 per cent sodium glycerophosphate did not induce clot retraction which was found to be absent both in the glycerophosphated and homologous control blood sample at the end of forty-eight hours. Also worthy of mention is the confirmation of the fact, previously noted, that blood samples containing completely anticoagulant concentrations of glycerophosphate, after a month of icebox storage, show barely a tinge of hemolysis.

SUMMARY

The addition of sodium glycerophosphate to shed blood in concentrations of 2 per cent or over completely prevents coagulation. Inhibition of coagulation is usually evinced by concentrations of 0.10 per cent and may be evinced in some samples by as little as 0.04 per cent. Concentrations which permit clotting cause an acceleration of clot retraction.

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BLUE FLUORESCENT SUBSTANCE IN URINE

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SOME of the carcinogenic hydrocarbons have very distinctive fluorescent spectra. These spectra consist of sharp bands in the blue region. This property has aided in the detection and isolation of these substances. The separation of benzpyrene from coal tar by Cook and co-workers¹ is a good example of this kind of investigation.

In the present paper is reported the detection of a substance in urine which has two bands in its fluorescent spectrum, with wave lengths of approximately 4100 Å and 4400 Å. Furthermore, this fluorescent property has allowed the substance to be traced through several chemical reactions. The physical and chemical properties thus far determined indicate that this substance is closely related to known carcinogenic agents.

The concentration of the substance was performed in such a manner that chemical changes in the urine would be avoided. The urine was placed in a low-temperature evaporator and dried from the frozen state. The construction of this evaporator already has been described.²

The residue was washed twice with benzene. The benzene washings from 100 liters of urine were collected and the benzene removed in the evaporator. This yielded a dark fatty residue, which was chilled in an ice bath and washed with ethyl alcohol. At this temperature the alcohol dissolved little of the fat but removed the fluorescent material, giving a dark solution.

The alcoholic solution was then reacted with paracarboxyphenylhydrazine. The latter is a ketone reagent that does not combine with the fluorescent substance but does combine with other substances that are present. One gram of paracarboxyphenylhydrazine was added to 100 c.c. of the alcoholic solution, and the flask, fitted with a reflux condenser, was placed in boiling water for one hour. The contents of the flask were then evaporated to dryness in the evaporator.

Two hundred cubic centimeters of 4 per cent potassium carbonate were added to the residue, and the mixture was heated gently for thirty minutes. This formed the potassium salt of the excess paracarboxyphenylhydrazine and all compounds formed from its reaction.

The water mixture was reduced to dryness in the evaporator and the residue washed with benzene. This benzene solution was then evaporated to dryness producing a few milligrams of oily material. This is the purest state in which the fluorescent substance has been produced.

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The fluorescent substance has been observed in all samples of urine that have been examined. Calf urine is the richest source thus far found. In the case of calf urine, the bands are clearly visible in the fluorescent spectrum of the first benzene washings, showing that this substance is native to the urine. With human urine, it is probably necessary to carry through the chemical reactions outlined before the bands become visible.

Molecules of this type may have a bond structure that will form an addition compound with maleic anhydride. This Diels-Alder reaction is exhibited by methylcholanthrene.³ The addition of maleic anhydride to a fluorescing solution of methylcholanthrene quenches the fluorescence instantly. The same effect is obtained with the unknown substance. It will be noted that the unknown substance is soluble in benzene and does not react with alkalis, paracarboxyphenylhydrazine, or digitonin.



Fig. 1.—Fluorescent spectrograms. 1, Methylcholanthrene; 2, unknown substance.

The fluorescent properties of a considerable number of biologic substances have been examined. None of them correspond to the unknown substance. From our experience, a natural product giving fluorescent bands in the blue would be a very rare material.

It is thought that this is a new substance whose nature should be determined regardless of its relation to cancer. A few mice were injected with the substance without causing tumors. However, this work may not be conclusive.

SPECTROSCOPIC EXAMINATION

The fluorescent light from the solutions is weak, so that it is wise to provide a strong source of ultraviolet light and photograph the spectrum with a spectrograph of large aperture. Apparatus that had been used previously for the fluorescence of porphyrins⁴ was readily adapted to the present work.

The light source was an arc of National "C" carbons operated at 40 amperes. A Corning filter No. 586 transmitted a narrow band of ultraviolet light around 3800 Å. A water cell placed between the arc and the filter allowed the arc to be operated within 8 cm. of the solution. The fluorescing solution was held in a test tube placed directly against the slit of a Steinheil spectrograph. Exposure times on the plates were about five minutes each.

Commercial solvents are likely to contain sufficient fluorescent material to obscure the results. Satisfactory benzene was obtained by washing the commercial product with concentrated sulfuric acid and then distilling twice the

benzene from the frozen state in the evaporator. The ethyl alcohol was a product purified in the Department of Biochemistry.

The present results were obtained over a period of time before their interruption by the war. The work was carried as far as the available resources would allow. The Department of Physics kindly made the Steinheil spectrograph available. It is also a pleasure to acknowledge valuable suggestions contributed by Dr. Rosalind Klass and Mrs. H. C. Brown, who have worked on the problem.

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LABORATORY METHODS

A SIMPLE METHOD FOR ASSURING ACCURACY IN DETERMINATION OF OXYGEN CONSUMPTION BY THE BENEDICT- ROTH APPARATUS

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IN CASES where breathing is irregular it is difficult to trace the "oxygen line" accurately. In some graphs two different lines, representing widely varying oxygen consumptions, can be traced, either of which can be considered the correct one. To obviate this difficulty, a simple method has been devised; it is based on the following considerations: The lung and spirometer form a closed system from which oxygen is removed by absorption through the lung. This system has two compartments, the respiratory tract and the spirometer. At each respiratory movement the relative proportion of gas in each compartment varies; therefore, in order to appreciate the total decrease in volume, it is necessary to measure the gas contained in the spirometer at the same moment of the respiratory cycle. Usually this is done by tracing a line through the points corresponding to normal expiration, when the lung contains only reserve (supplemental) and residual air. The volume of reserve air varies with the depth of expiration, a fact which introduces a variable in the proportion of gas contained in the spirometer, having no relation to the total volume. The residual air, on the other hand, remains constant in the conditions of the experiment; therefore, if only this part of the total volume of gas remains in the respiratory tract, variations in the spirometer volume will correspond accurately to changes in total volume; that is, to oxygen consumption.

PROCEDURE

While the patient is breathing normally into the spirometer, he is instructed to make a deep expiration. It is important to assure the maximum possible emptying of the lung, so that all the reserve (supplemental) air will be expelled into the spirometer; for this the subject must be stimulated to go on expelling air until the expiratory line flattens out. This is repeated once or twice at two- to six-minute intervals. The "oxygen line" is traced by joining the points of extreme expiration. In Figs. 1 and 2 the "oxygen line" obtained by tracing it at the end of normal expiration was parallel to that obtained by joining the points of extreme expiration. Also shown in Fig. 2 is the constancy of residual air, since the three points of extreme expiration are in a straight line. In both cases, as in many others with regular respiratory rhythm, there was no difference in the oxygen consumption values determined by both methods.

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Out of forty-two cases of normal, hyperthyroid, and hypothyroid subjects taken at random, the difference between the oxygen values given by both methods was less than ± 0.5 cal. per m^2h in twenty-seven; it was between 0.5 and 1 cal. in six cases, 1 and 2 cal. in five cases, 2 and 3 cal. in three cases, and in one case the method proposed gave a figure 4 cal. below that obtained by the usual method (Fig. 3).

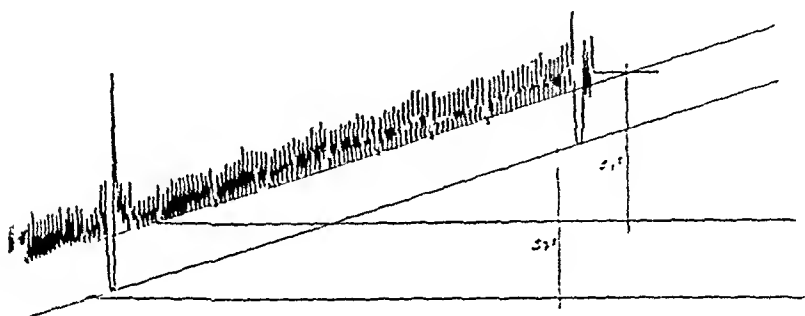


Fig. 1.

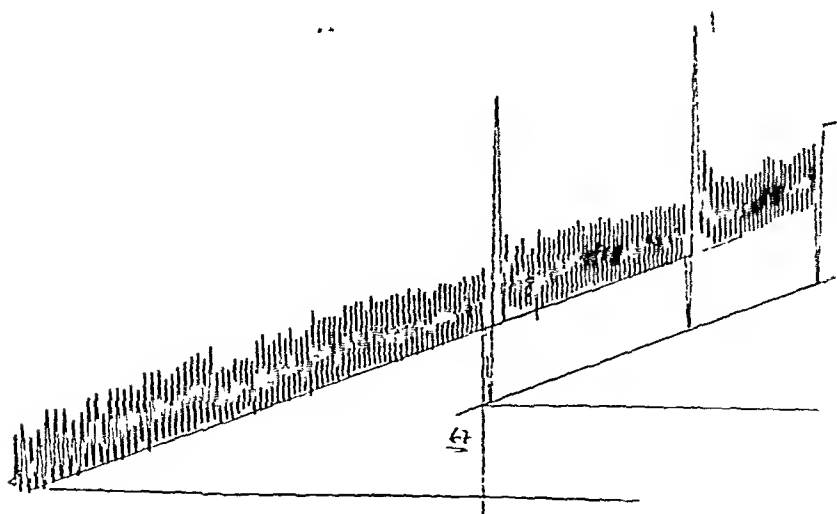


Fig. 2.

In Fig. 4 is shown a respiratory curve that cannot be used to determine oxygen consumption by the normal method because of irregular respiration. The oxygen consumption as determined by the method proposed was 34.8 cal.

m^2h and 35.5 cal. m^2h by the Tissot-Maldane open circuit method. A similar agreement between the results obtained by both these methods was registered in five cases.

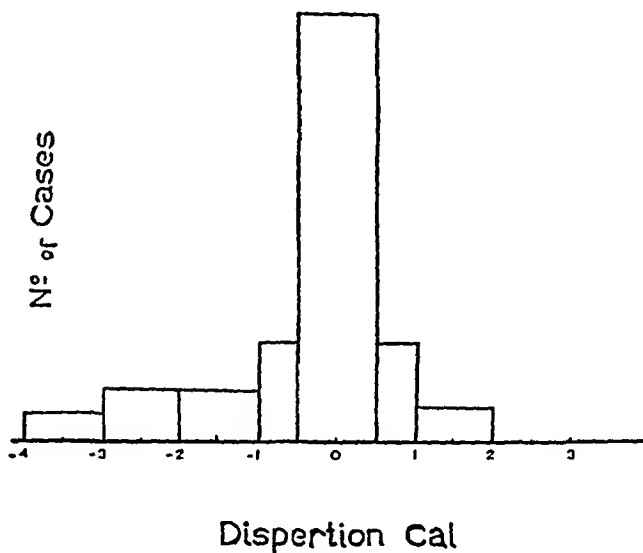


Fig. 3.

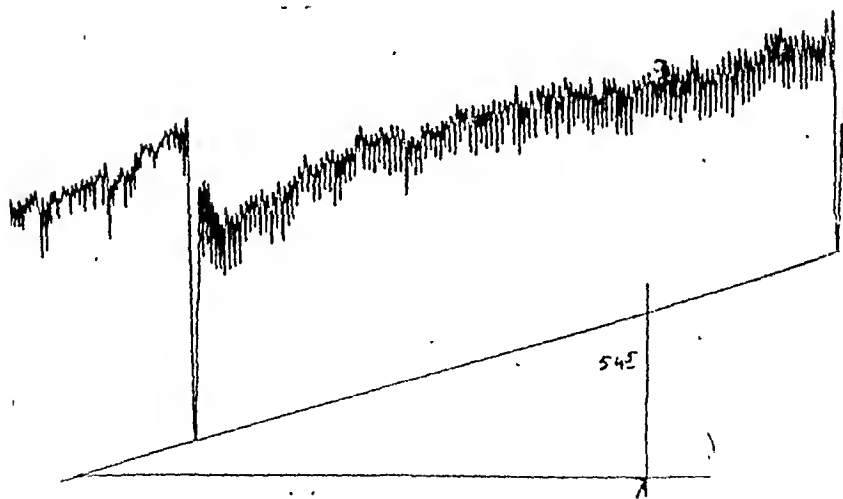


Fig. 4.

SUMMARY

The "oxygen line" in the Benedict-Roth basal metabolism method can be traced at the end of extreme expiration with greater accuracy than at the end of normal expiration, since the volume of residual air remains constant; while the volume of residual plus reserve (supplemental) air varies with the depth of respiration. Accurate oxygen consumption determinations can be made with this method in cases with irregular respiratory rhythm in which by the usual method it is impossible to do; this often occurs in cases of hyperthyroidism in which basal metabolic rate determinations are of primary importance.

A SIMPLIFIED METHOD FOR THE ESTIMATION OF SODIUM

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THE method of estimating the sodium content of urine or blood as described in this paper was developed in the course of a study of the effectiveness of various diuretics. In many of the published articles chloride excretion has been used as an index of sodium loss, but there have been relatively few studies on the ability of various diuretics to cause excretion of sodium. An investigation of the diuretic action of ammonium chloride made it necessary to use a method of determining sodium rather than chloride. A preliminary trial of several of the sodium methods indicated the need of simplification before they could be adapted to routine use. To meet this need a simplified technique was developed and is presented herein as a procedure worthy of use as a routine test.

Sodium was first determined quantitatively by precipitation with uranyl zinc acetate by Barber and Kolthoff.^{1, 2} Butler and Tuthill³ applied this method to biologic solutions and found that interfering substances, such as proteins and phosphates, could be eliminated before the sodium was precipitated. Salit⁴ modified the procedure by the addition of alcohol to obtain a quantitative precipitation of the triple salt. The triple salt was then caused to react with potassium ferrocyanide to develop a reddish brown color, the intensity of which was determined in a colorimeter. In our experience, the pH of the solution and the time of color development had to be closely controlled. Weinbach⁵ adopted the alcoholic precipitation method of Salit but preferred to titrate the triple salt against 0.02 N sodium hydroxide. Since amphoteric hydroxides were formed in this titration, the end point was not sharp.

Arnold and Pray⁶ described a colorimetric method for the determination of sodium. However, the color change in their method required the use of a spectrophotometer. Caley and Foulk⁷ used uranyl magnesium acetate to precipitate sodium as a triple salt. They found that when the triple salt was dissolved, the color intensity of the solution was proportional to the concentration. It occurred to us that since the uranyl ion made the reagent a brilliant yellow, the supernatant solution should lose color in proportion to the amount of triple salt that was precipitated. This possibility was realized in subsequent tests, so the method described here is based on the color intensity of the reagent after precipitation of the triple salt. Use of the supernatant reagent has the advantage of eliminating the steps of washing the triple salt and any subsequent procedures of weighing, transfer, color development, or titration.

Reagents.—The uranyl zinc acetate was prepared in the proportions given by Weinbach. However, it was found that the uranyl acetate (77 Gm.) dis-

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solved more quickly in 800 c.c. of water plus 14 c.c. glacial acetic acid. The zinc acetate (231 Gm.) was then added in small portions which dissolved very quickly. The final 7 c.c. of acetic acid was added and the solution made up nearly to volume. After cooling and standing overnight it was made up to final volume (1 liter). This reagent was equally as satisfactory as that prepared by dissolving the uranyl and zinc acetates separately and combining the hot saturated solutions.

Standard solutions of chemically pure sodium chloride were made up over a range of 1.0 to 10.0 mg. per cubic centimeter, at 1 mg. intervals. These were needed to prepare the colorimetric absorption curve and to use as checks in subsequent determinations of unknowns. Since sodium chloride was used as a standard, we have expressed our values as equivalents of sodium chloride rather than sodium. Other salts of sodium such as the carbonate, acetate, or citrate gave quantitative sodium equivalents.

The colorimetric determinations were made with a Fisher electrophotometer using the blue filter No. 425 and the 3 c.c. micro cells.

Preparation of Urine.—The urine should be refrigerated for several hours and then filtered before using. Salit reported that the bulk of the phosphates and urates can be precipitated by refrigeration. In our experience, if the urine was alkaline or was made alkaline by allowing ammonia to bubble through it, the phosphates were so completely precipitated by refrigeration that the addition of calcium hydroxide was not necessary. If albumin was present it was removed by addition of mercuric chloride or trichloroacetic acid.

Preparation of Blood or Serum.—An equal volume of 20 per cent trichloroacetic acid should be added slowly and stirred vigorously to break up the clumps. This should be centrifuged and then the clear supernatant fluid used.

METHOD

Pipette 1 c.c. clarified urine, deproteinized serum, or sodium-containing solution into a 15 c.c. centrifuge tube.

Add 4 c.c. uranyl zinc reagent, freshly filtered.

Add 2 c.c. 95 per cent alcohol. (Use volumetric pipettes for these three solutions.)

Stir or invert the tubes to obtain thorough mixing and then allow to stand for twenty to thirty minutes.

Centrifuge. Decant the clear supernatant into the colorimeter absorption cell. If there is a pellicle of floating crystals, it may be eliminated by decanting the first few drops and then pouring the rest of the solution into the absorption cell.

Determine the color intensity with the photometer.

When urine is used as the unknown, there must be a correction for the yellow color of the urochrome. This is done by making up a blank tube with 1 c.c. of the urine specimen, 4 c.c. of water, and 2 c.c. of alcohol. The photometer reading of this blank is subtracted from the reading of the uranyl solution containing the urine.

The sodium content of the unknown is determined by interpolating the corrected photometer reading on the absorption curve prepared from the standard sodium solutions. The type of curve obtained is illustrated in Fig. 1. The scale values will be different for each photometer and for the absorption cells unless they are perfectly matched.

One or two standard solutions and a blank have been included with each determination to be certain that the conditions of the test and the solutions have not changed. The greatest variations observed in multiple determinations on standard solutions are indicated by the bars above and below the curve in Fig. 1. An example of the colorimeter readings and the sodium values for two urine specimens is given in Table I.

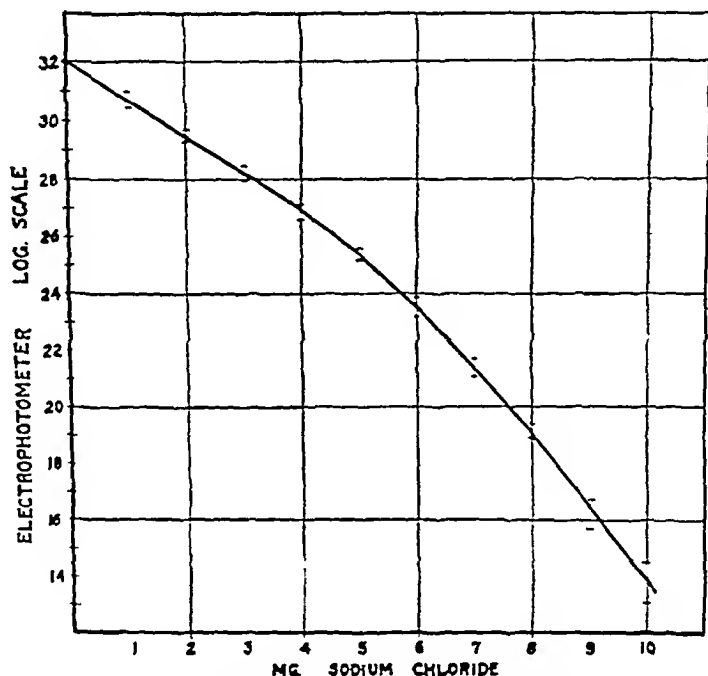


Fig. 1.—Absorption curve of uranyl zinc acetate reagent after precipitation by increasing quantities of sodium chloride. Obtained with a Fisher electrophotometer using blue filter No. 425 and 3 c.c. micro cells.

This colorimetric method was checked gravimetrically by mixing the solutions in a Jena filter and washing and drying the triple salt and then determining its weight. The method was that of Butler and Tuthill except for the proportion of the solutions and the addition of alcohol. The results of one trial using blood serum and standard solutions of sodium chloride are shown in Table II. In general, there was close agreement of values for each solution.

When sodium chloride values are less than 1.5 mg. per cubic centimeter, the difference between colorimeter readings is only 0.6 to 0.8 for 0.5 mg. differences in concentration. The absorption cells that were supplied with the electrophotometer varied as much as 0.6, so it was necessary to use the same cell for each color reading to detect small differences. To insure that the cell was in the

TABLE I. EXAMPLE OF PHOTOMETRIC READINGS OBTAINED ON URINE SAMPLES AND STANDARD SOLUTIONS OF SODIUM CHLORIDE AFTER TREATMENT WITH URANYL REAGENT AND CALCULATED SODIUM CHLORIDE CONCENTRATION IN URINES

	PHOTOMETER READING	COLOR CORRECTION	CORRECTED READING	CALCULATED NaCl (MG./C.C.)	
Urine No. 1	28.8	2.8	26.0	4.6	
Urine No. 2	24.4	6.3	18.1	8.4	
Blank	32.6				
Standard NaCl		Observations made to be certain that conditions of the test and the solutions have not changed			
5 mg./c.c.	25.3				
10 mg./c.c.	14.6				

TABLE II. COMPARISON OF GRAVIMETRIC AND PHOTOMETRIC ESTIMATIONS OF SODIUM CHLORIDE IN BLOOD SERUM AND IN STANDARD SOLUTIONS

SOLUTIONS TESTED	OBSERVATIONS		CALCULATED NaCl (MG./C.C.)	
	GRAVIMETRIC MG.	PHOTOMETRIC READING	GRAVIMETRIC	PHOTOMETRIC
Serum	114.0	26.6	8.50	8.40
Standard solutions (mg./c.c.)				
10.0	267.0	13.7	9.97	10.05
5.0	138.0	24.8	5.10	5.30
1.5	40.2	30.3	1.50	1.50
1.0	27.5	31.1	1.02	0.90
0.5	13.6	31.7	0.50	0.45

same position for each reading, a point was heated in a Bunsen flame, and a small bleb was blown in the side of the cell. This bleb fitted into a notch that had been cut in the carrier and assured exact orientation. By rinsing the cell with small portions of the solution to be read, any significant dilution by the remains of the previous solution was avoided and duplicate samples usually checked within 0.2 on the photometer scale. The curve is steeper when sodium chloride values exceed 4 mg. per cubic centimeter and differences in readings are more significant. Since, in our experience with urines, the sodium chloride concentration never exceeded 10 mg. per cubic centimeter, the curve was not extended beyond that point. Casual observations indicate that it can be used at least up to 15 mg. per cubic centimeter. It cannot be used to the theoretical zero because the yellow triple salt is soluble when the concentration of the reagent is reduced beyond a critical level.

SUMMARY

A simplification of the uranyl zinc method for determining sodium is described.

The method has been found suitable for the routine testing of urine and blood.

The method can be used when sodium chloride concentrations are as low as 0.5 mg. per cubic centimeter. Estimations by this colorimetric method have usually checked within 2 per cent of those obtained by the gravimetric method.

The author is indebted to Miss Donna MacLachlan and Miss Amanda Piper, for technical assistance in testing this method.

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A DIRECT PHOTOELECTRIC METHOD FOR THE DETERMINATION OF SERUM CALCIUM

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ONE of the disadvantages of the titrimetric methods for the determination of serum calcium with potassium permanganate is the difficulty encountered in reading the end point when the usual visual procedures are used. It should be possible to avoid this disadvantage by the application of colorimetric or spectrophotometric techniques to the analysis of serum calcium and at the same time to effect a considerable saving of time. Colorimetric methods for calcium have been based on the final precipitation of the element, as the alizarinate,¹ phosphate,²⁻⁴ tungstate,⁵ or oxalate.⁶ More recently a method has been developed for the determination of calcium by precipitation as the oxalate and the spectrophotometric measurement of the permanganate.⁷

Some of the afore-mentioned methods, as colorimetric methods, have been used to a considerable extent for the special purpose for which they were developed. Their general limitations are not well established, and their applicability to the determination of serum calcium has been studied in only a few instances.

Sendroy⁶ developed a photoelectric method for the determination of serum calcium based on the reduction of ceric sulfate and the measurement of the yellow color after the addition of excess potassium iodide. This method appears to give accurate results in the hands of an experienced analyst, however, it is rather laborious for the routine analysis of large numbers of samples, and it employs indirect equivalent relations and calculations.

The application of the permanganate method to the photoelectric determination of serum calcium appeared to offer several advantages over existing methods for serum calcium. The method presented in this paper is based on the precipitation of calcium as the oxalate and the photoelectric or spectrophotometric measurement of the amount of potassium permanganate reduced by using an excess of this reagent. By this method serum calcium may be determined with a high degree of accuracy on a relatively large number of samples with any one of a number of modern photoelectric colorimeters or spectrophotometers.

Reagents.—

Sodium oxalate solution. (Prepared by dissolving approximately 6 Gm. of sodium oxalate in 1 liter of distilled water.)

2 per cent ammonium hydroxide.

Approximately 5 N sulfuric acid.

From the Nutrition Laboratory, Agricultural Experiment Station, Agricultural and Mechanical College of Texas.

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Standard potassium permanganate solution—approximately 0.01 N. (This may be used without standardizing provided a calibration curve is prepared with each new solution. The stability of the potassium permanganate can be conveniently checked at intervals with standard calcium oxalate. It may, however, be more convenient to prepare one standard curve for a specific normality and thereafter to adjust each new solution to that particular normality.)

Procedure.—To a 15 c.c. graduated conical centrifuge tube add 2 c.c. of serum that is free from erythrocytes plus 1 c.c. of the solution of sodium oxalate. Mix and allow to stand overnight to insure complete precipitation of the calcium oxalate. Centrifuge for five minutes at about 2,600 revolutions per minute. This packs the precipitate of calcium oxalate firmly enough to permit siphoning off all but about 0.25 c.c. of the serum and excess oxalate solution. This is conveniently done by using a capillary tube with an upturned tip. Resuspend the precipitate in the remaining solution and then add 5 c.c. of 2 per cent ammonium hydroxide from a pipette so that the sides of the tubes are rinsed to remove residual oxalate. Centrifuge at about 2,600 r.p.m. for five minutes. Repeat the washing procedure two more times or until all residual oxalate has been removed.

After washing, dissolve the precipitate in 5 c.c. of 5 N sulfuric acid. Place the centrifuge tubes containing the dissolved calcium oxalate in a boiling water bath for five or six minutes. Add exactly 2 c.c. of the potassium permanganate solution and dilute to 10 cubic centimeters. Let stand for five minutes, but no longer than fifteen minutes, before making spectrophotometric or colorimetric readings at 530 millimicrons.

Calibration Curve.—Calcium sulfate is used for preparing the calibration curve. A series of tubes is set up containing the following amounts of calcium: 0.10, 0.14, 0.18, 0.20, 0.22, 0.26, 0.30 and 0.34 milligram. The standard is then treated exactly as described for the serum. The density or transmittance, depending on which reading is taken, is plotted along the ordinate and the amounts of calcium along the abscissa of the graph. Calcium levels ranging from 5.0 to 17.0 mg. per 100 c.c. of serum can be read from the graph. Density measurements made with the Beckman spectrophotometer and transmittance measurements made with an Evelyn photoelectric colorimeter showed that Beer's law holds precisely over this range.

The accuracy of the procedure and the standard curve prepared with calcium sulfate may be verified with a standard calcium oxalate solution. The standard calcium oxalate is made up in 5 N sulfuric acid and added to the centrifuge tubes at calcium concentrations ranging from 0.1 to 0.34 milligram. Additional 5 N sulfuric acid is added to bring the volume in each tube up to 5 cubic centimeters. These are then treated as described for the serum following the solution of the oxalate. The spectrophotometric or colorimetric readings should check exactly with the readings for the calcium sulfate standards. The constancy of the potassium permanganate solution can be conveniently checked from time to time by the standard calcium oxalate.

Wave Length.—Transmittance minima for dilute permanganate solutions have been reported ranging from 526 to 530 millimicrons. Scott and Johnson⁷ using a Coleman spectrophotometer reported that the transmittance minimum for dilute permanganate was 529 millimicrons. Using a Beckman quartz spectrophotometer, we have found that dilute permanganate solutions containing the same amount of acid as in the procedure for calcium have a maximum density at 529 to 530 millimicrons. With the Evelyn photoelectric colorimeter we have used a filter with transmittance maxima at 540 millimicrons.

TABLE I. RECOVERY OF CALCIUM ADDED TO SERUM

SAMPLE	VOLUME OF SERUM (C.C.)	CALCIUM CONTENT OF SAMPLE (γ)	CALCIUM ADDED (γ)	TOTAL (γ)	FOUND	
					(γ)	(%)
1	2.0	252	80	332	334	100.6
2	2.0	213	80	293	301	102.8
3	2.0	226	80	306	301	98.4
4	2.0	238	100	338	352	104.1
6	2.0	250	80	330	328	99.4
6	1.6	222	64	286	268	93.7
6	1.2	164	48	212	200	94.4
7	2.0	234	83	317	332	104.7
8	2.0	242	83	325	343	105.5
8	1.6	221	66	287	281	97.9
9	2.0	257	83	340	358	105.5
9	1.6	220	66	286	271	94.7
10	1.2	179	48	227	235	103.5
11	2.0	262	80	342	345	100.9
11	1.2	176	48	224	235	104.9
12	2.0	240	80	320	331	103.4
12	1.6	218	64	282	276	97.9
13	2.0	201	80	281	283	100.7
14	2.0	240	80	320	319	99.7
14	1.6	195	64	259	252	97.3
14	1.2	156	48	204	209	102.4
15	2.0	229	80	309	309	100.0
15	1.6	195	64	259	277	106.9
15	1.2	154	48	202	209	103.5
16	2.0	256	80	336	340	101.2
16	1.6	241	64	305	301	98.7
16	1.2	164	48	242	245	101.2
Average						100.9
Standard deviation						3.6

Recovery Experiments.—Serum was obtained from various species by allowing the freshly drawn blood to clot, storing it in the refrigerator for a few hours. Each sample of serum was analyzed for calcium according to the technique described. A known amount of calcium, as calcium sulfate, was then added to the serum of each sample and analysis run to measure the recovery. These data showing the recovery of calcium added to serum are presented in Table I. The mean recovery of added calcium measured on the Beckman spectrophotometer was 100.9 per cent. The relatively small variation in the per cent recovery is apparent from the standard deviation of ± 3.6 . There is no evidence of any constant error in the method, as the per cent of calcium recovered for individual samples of serum was neither consistently above nor below the expected value.

Comparison of Values Obtained With Beckman and Evelyn Instruments.—Samples of serum from eighteen different animals were analyzed for calcium. Measurements were made on a Beckman spectrophotometer and on an Evelyn photoelectric colorimeter. Expressed on the basis of milligrams of calcium per 100 c.c. of serum, the mean value as determined with the Beckman spectrophotometer was 11.7 and with the Evelyn photoelectric colorimeter, 11.8.

Comparison of Values Obtained Spectrophotometrically and by Electrometric Titration.—The values for serum calcium by the spectrophotometric procedure were compared with the values obtained by electrometric titration of the oxalic acid with 0.01 N potassium permanganate using a Fisher titrimeter. Each sample of serum was set up in quadruplicate and treated according to the procedure outlined, except that the oxalic acid of two of the tubes was measured by electrometric titration with standard permanganate solution. The values for serum calcium as measured spectrophotometrically and by electrometric titration are recorded in Table II. The values obtained by the two procedures agree very well for each sample, the maximum variation being 0.4 mg. of calcium per 100 c.c. of serum. While only the averages for the duplicates are presented in Table II, it is significant that the magnitude of the variation between duplicate determinations was of the same order as the variation between the two procedures.

TABLE II. COMPARISON OF SERUM CALCIUM VALUES DETERMINED SPECTROPHOTOMETRICALLY AND BY ELECTROMETRIC TITRATION

SAMPLE	SPECTROPHOTOMETRIC (MG. PER 100 C.C.)	ELECTROMETRIC TITRATION (MG. PER 100 C.C.)
1	11.0	10.9
2	11.4	11.2
3	12.1	12.0
4	11.1	11.2
5	12.7	12.7
6	14.6	14.4
7	13.0	13.4
8	11.6	11.4
9	11.5	11.3
10	11.2	11.1
11	11.4	11.8
12	10.7	11.0
13	12.0	12.0
14	11.0	10.9
15	12.5	12.2
Average	11.8	11.8

SUMMARY

A method is described for the spectrophotometric determination of serum calcium. The calcium is precipitated as the oxalate and measured by the extent of the reduction of a constant amount of potassium permanganate by means of a spectrophotometer or photoelectric colorimeter. The values for serum calcium by this procedure agreed very well with the values obtained by electrometric titration with standard permanganate solution.

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A RAPID BEDSIDE TEST FOR THE DETECTION OF HYPOGLYCEMIA

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IN CERTAIN cases the diagnosis of hypoglycemic reaction can be quickly made from a suggestive history and consistent physical findings. Unfortunately, not all cases present such a simple problem; the history is often unobtainable or misleading, and the physical signs are inconclusive. A quantitative determination of the blood glucose concentration usually requires (in an average, reasonably well-equipped hospital) twenty minutes or more. (An analysis can, of course, be made more quickly if other analyses are already in progress.)

Because of deleterious effects, especially upon the central nervous system, it is imperative that the question of hypoglycemia in a patient with suggestive findings be settled rapidly. This fact has given rise to the practice of administering glucose intravenously as a therapeutic test. In an appreciable number of cases, this is a rational and useful method of making the diagnosis and initiating therapy.

But the need for an alternative procedure is apparent from the following considerations. The number of patients admitted in unexplained coma or with other findings suggestive of hypoglycemia (deserving, therefore, immediate investigation) is large in proportion to the number who are found actually to have hypoglycemia. A therapeutic test requires a heavy dose of intravenous glucose, since at times a surprisingly large amount must be given rapidly to produce an effect in a patient with hypoglycemia. A patient admitted to the Rhode Island Hospital required 70 c.c. of 50 per cent glucose solution intravenously before showing any response. The only practical way of giving the needed amount is to use a very concentrated solution. One cannot draw conclusions from failure to obtain a response to a small amount of the concentrated solution. Since large amounts of concentrated, hypertonic glucose solutions are definitely dehydrating, hypoglycemia should be investigated by some other method in the case of poorly hydrated patients with suggestive findings, not an uncommon situation both in patients with hypoglycemia (for example, Addisonian) and normoglycemia.

The method described here utilizes commercial powder (containing oxidized bismuth and alkali) employed to detect glucose in urine. Such powder turns gray or black when exposed to a solution containing a sufficient amount of reducing agent. Whole blood is used; the proteins are precipitated by copper. Since much of the time consumed in blood glucose determinations is taken up by the gravity filtration of the viscous mixture, a simple method of pressure filtration is substituted here.

From the Rhode Island Hospital, Providence, R. I.
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A short test tube (tubes approximately 7 by 1.5 cm. are satisfactory), containing 200 mg. of dry $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, is marked to contain 5 c.c. of whole blood. Blood taken by venipuncture is introduced directly into the tube to the 5 c.c. mark, and sufficient 3 N NaOH is added from a dropper or pipette to make the mixture just alkaline to litmus; 0.4 c.c. (or 6 drops from the average dropper) is required. It has been found that the reaction can be carried out most rapidly by using a dropper which has already been calibrated in previous tests, with the aid of litmus. Once the required number of drops from a given dropper is known, it can be used without the necessity of checking with litmus, and alkalization can be performed more rapidly and conveniently than with a graduated pipette.

The mixture is stirred with a small wooden reamer or thin glass rod, the tube corked, and mixing completed by shaking. Part of the mixture is then poured into the barrel of a 10 c.c. syringe, at the bottom of which two or three disks of filter paper have been placed. The disks must fit the internal diameter of the syringe closely. (Disks used in penicillin assay have been found satisfactory.) The plunger is then inserted and pushed down the barrel, compressing the mixture, so that a clear filtrate rapidly escapes through the nozzle. The first 2 or 3 drops of filtrate are discarded, and the next drop is allowed to fall on a *large excess* of Galatest powder. If within a period of forty-five seconds, a definite gray or black color appears, the reaction is considered normal. Failure of a definite gray or black to appear constitutes an abnormal (hypoglycemic) result. Since any filtrate may discolor the powder brown or yellow, an unequivocal (but not necessarily intense) black or gray must develop, if the reaction is to be considered normal. However, if the test is performed against a white background, this distinction is usually very easy to make, even if only a faint gray color develops.

In developing this test, whole oxalated blood was incubated for twenty-four hours at 37° Centigrade. Various amounts of glucose were then added to portions of the autoglycolysed blood. The artificially set blood glucose concentrations were checked by the method of Benedict.*† At the Rhode Island Hospital this method has given a normal range of 50 to 100 mg. per 100 cubic centimeters. For all practical purposes, a value less than 50 mg. per cent, or, at any rate, less than 45 mg. per cent, may be considered abnormal. In a series of bloods, with values set, as previously described, from 8 to 67 mg. per cent, the rapid method outlined herein gave abnormal reactions with all bloods containing 44 mg. per cent or less and normal reactions with all bloods containing 53 mg. per cent or more. Repetition of the tests substantiated this.

The speed with which the blood glucose decreases is a factor in determining how low the concentration may fall before symptoms supervene; therefore, an occasional patient whose low value is not actually the cause of his symptoms may be diagnosed as abnormal by this test. In such a case, however, the danger

*Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and of Saccharoids (Nonfermentable Carbohydrate Reducing Substances), J. Biol. Chem. 92: 19-41, 1931.

†Methods which determine blood "glucose" actually determine glucose plus a certain percentage of nonglucose reducing substances. The latter represent only a few milligrams per cent in the Benedict method.

point would be close, so the diagnosis and resultant therapy could not be considered seriously in error. A value extremely close to 50 mg. per cent is difficult to read; a patient whose blood yields such a value may receive needless glucose, but such cases should be rare. The test as described here will detect all cases with blood glucose sufficiently low to cause serious effects, while ruling out the vast majority of patients with normal or elevated values.

The apparatus needed, prepared in advance, can conveniently be kept in a small container on the ward and the test carried out at the bedside. Under these circumstances, performance requires three to three and one-half minutes from the time blood is drawn from a vein.

The Galatest brand of urine-testing powder was used because it was readily available at the time of these experiments. Nothing written in this article should be construed as a specific recommendation for this particular brand. It cannot be stated now whether any other powder of similar composition would be superior or inferior.

In conclusion, it should be stressed that this test is useful solely to discover quickly whether hypoglycemia is present in a patient with suggestive findings for whom the needless intravenous administration of a large amount of glucose would be harmful. It is intended to supplement, but certainly not to replace, quantitative blood glucose determinations, which are indispensable in the proper study of hypoglycemia and its course.

SUMMARY

1. A method whereby the diagnosis of hypoglycemia can be made or ruled out in three to three and one-half minutes is described.
2. Mention is made of occasions where this method is preferable to the use of a therapeutic test with intravenous glucose solution.

AN ABBREVIATED SPECTROPHOTOMETRIC TECHNIQUE FOR DETERMINING THE OPTIMAL CONCENTRATION OF AMBOCEPTOR

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WITH THE TECHNICAL ASSISTANCE OF REBECCA GOODMAN
AND HELEN CONWAY

THE optimal concentration of amboceptor has been defined as that beyond which further increase fails to enhance appreciably the hemolytic activity of complement.^{1a} The demonstration of this optimum depends largely upon the accuracy with which changes in the activity of complement are measured and accordingly follows the application of a quantitative method in the titration of complement for fixation studies.² The method in question employs the end point of 50 per cent hemolysis.

Use of the 50 per cent end point demands accuracy in determining the percentage of hemolysis in titrations. The required degree of precision has been obtained through the use of prepared color standards.^{1b} More recently, adaptations of the spectrophotometer have contributed to the accuracy and facility of the determination,^{3, 4} and the employment of this instrument in conjunction with a simple graphic method has permitted the quantitative determination of the 50 per cent unit of complement without constructing the curve of hemolysis.⁴ Continued studies at this laboratory have included the application of similar methods to the standardization of amboceptor. As a result, there has been developed a simplified technique for determining the optimal concentration of the amboceptor. The procedure employs the spectrophotometer in measuring percentage of hemolysis, and a standard curve is applied in determining the 50 per cent unit of complement when varying concentrations of amboceptor are used. Since the method has yielded accurate and reproducible results in our hands, a description may be of some interest to those engaged in complement fixation studies.

MATERIALS AND METHODS

Spectrophotometer.—The Coleman Junior Clinical Spectrophotometer Model 6* is used. All measurements are made in terms of optical density at 580 millimicrons. The use of a 12 mm. adapter permits the employment of selected 12 by 75 mm. serologic tubes as cuvettes. Tubes of uniform internal diameter are selected on the basis of snug fit with a metal rod cut to 10 mm. diameter.

From the Division of Serology, Army Medical School, Army Medical Center.
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*Manufactured by the Coleman Electric Company, Maywood, Ill.

A total volume of 2.0 c.c. in these tubes is required to bring the level of the contents well above the slit through which light passes before striking the photoelectric cell. This volume represents twice that used in diagnostic tests at this laboratory and occasions the use of double volumes (2V) of complement, salt solution, amboceptor, and sheep's cells in spectrophotometric determinations. The result of a titration carried out under the latter conditions is transferable without correction to the conditions of the diagnostic tests employing unit volumes (V) of the reagents since, as in the amboceptor titration to be described, it is expressed directly in terms of the required concentration, or dilution, of the reagent titrated.

Diluent.—Salt solution, 0.85 per cent, buffered to pH 7.3 with 0.005 M phosphate is employed as diluent for all reagents. In practice, a stock solution containing 170.0 Gm. of NaCl, 2.7 Gm. of KH_2PO_4 , and 11.3 Gm. of Na_2HPO_4 to the liter is diluted 1:20 in distilled water before use.

Sheep's Red Blood Cells.—A 2 per cent suspension is prepared from blood preserved by aseptic collection into an equal volume of modified Alsever's solution.⁵ The required volume of blood is washed three times with salt solution in 15 c.c. centrifuge tubes. An International centrifuge with head No. 240 is used. After a final centrifugation for ten minutes at 2,000 r.p.m., the volume of packed cells is read, and the 2 per cent suspension is prepared by diluting to 50 volumes with salt solution. It is then standardized spectrophotometrically. The suspension is mixed thoroughly by rotation, after which a 0.4 c.c. sample is measured accurately into a 12 by 75 mm. tube and lysed by the addition of 1.6 c.c. of distilled water. The tube is spun for five minutes at 2,000 r.p.m., and the optical density is determined at 580 $\text{m}\mu$ against a tube of water as a blank. Suspensions yielding an optical density of 0.500 ± 0.01 are accepted. Corrections to this reading are made by adding or removing salt solution, the required final volume of the suspension being determined according to the relation

$$V_2 = \frac{V_1 \text{OD}_1}{0.500}$$

where V_1 is the volume of the suspension yielding the observed optical density OD_1 , and V_2 is the final volume required to yield an optical density of 0.500. Salt solution is removed, when necessary, by spinning a suitable amount of the suspension, removing the required volume of supernatant salt solution, and resuspending the remaining supernate and cells in the parent 2 per cent suspension.

In the Coleman Junior Spectrophotometer, using the standardized 12 by 75 mm. tubes, a mean optical density of 0.500 at 580 $\text{m}\mu$ has been found to correspond with a cell concentration of 500,000 per cubic millimeter. If other instruments or cuvettes are employed, the mean optical density equivalent to this cell count should be redetermined.

Amboceptor.—Amboceptor produced by the immunization of rabbits is preserved by lyophilization or by the addition of an equal volume of neutral glycerol, chemically pure. A stock solution representing a 1:100 dilution of original serum is prepared by adding 99.0 c.c. of salt solution to 1.0 c.c. of

rehydrated serum, or 98.0 c.c. of salt solution to 2.0 c.c. of glycerolized serum. It may be stored safely at 3 to 6° C. for one week.

Complement.—Commercial, lyophilized guinea pig serum is used. The dried equivalent of 7.0 c.c. is rehydrated by the addition of 5.0 c.c. of the accompanying diluent which contains 2 per cent boric acid and 6 per cent sodium acetate. A stock 1:20 dilution sufficient for the day's tests is prepared by adding 19 volumes of salt solution to 1 volume of the rehydrated guinea pig serum.

Fresh, pooled guinea pig serum may be used with equally satisfactory results.

DETERMINATION OF THE OPTIMAL CONCENTRATION OF AMBOCEPTOR

Preparation of the Amboceptor Dilutions.—A 1:500 dilution of amboceptor is prepared by adding 8.0 c.c. of salt solution to 2.0 c.c. of the stock 1:100 dilution. According to our practice, an amboceptor showing an optimum at a dilution less than 1:500 is not considered of satisfactory titer. Varying quantities of 1:500 amboceptor and salt solution are combined as indicated in Table I to provide dilutions representing a suitable progression from 1:500 to 1:4,000. These dilutions are used to sensitize equal volumes of the cell suspension which have been measured accurately into separate tubes. It has been our custom to measure the 5.0 c.c. quantity of 1:500 amboceptor and corresponding volume of cells into separate 15 by 150 mm. tubes and the 1.6 c.c. quantities of the higher amboceptor dilutions and corresponding volumes of cells into separate 13 by 100 mm. tubes. The amboceptor dilutions are then poured into the respective tubes of cells; the two are mixed thoroughly by ten successive pourings, and at least ten minutes at room temperature are allowed for sensitization.⁶

TABLE I. PREPARATION OF AMBOCEPTOR DILUTIONS

	AMBOCEPTOR DILUTIONS						
	1:500	1:666	1:1000	1:1333	1:2000	1:2666	1:4000
Amboceptor 1:500 (c.c.)	5.0	1.2	0.8	0.6	0.4	0.3	0.2
Salt solution (c.c.)	0	0.4	0.8	1.0	1.2	1.3	1.4

Preparation of 1 Unit Complement.—Complement is titrated to the end point of 50 per cent hemolysis.⁴ A 1:100 dilution is prepared by adding 4.0 c.c. of salt solution to 1.0 c.c. of the stock 1:20 dilution of complement. It is titrated as indicated in Table II, using the cells that have been sensitized with 1:500 amboceptor. Carefully measured quantities of the reagents are added in the order given, and the contents of each tube are thoroughly mixed. After incubation for thirty minutes in the water bath at 37° C., all tubes of the titration are spun for ten minutes at 2,000 r.p.m., and the optical densities of their supernates are determined spectrophotometrically at 580 millimicrons. The galvanometer of the instrument is set at "0" optical density (100 per cent transmission) with Tube 10 in the adapter, and the optical densities of Tubes 1 to 9 are determined successively. Tubes 7 to 9 contain an excess of complement calculated to yield 100 per cent hemolysis. The mean of their three optical densities is taken as the standard of reference. The percentage of hemolysis in each of the first six

tubes is 100 times the quotient obtained by dividing its optical density by the mean value for 100 per cent hemolysis. The optical densities and corresponding percentages of hemolysis in Table II represent a typical result.

TABLE II. COMPLEMENT TITRATION

	TUBE									
	1	2	3	4	5	6	7	8	9	10
Stock 1:20 complement (c.c.)							0.40	0.40	0.40	
1:100 complement (c.c.)	0.30	0.35	0.40	0.45	0.50	0.55				
Salt solution (c.c.)	0.90	0.85	0.80	0.75	0.70	0.65	0.80	0.80	0.80	1.20
Cells sensitized with 1:500 amboceptor (c.c.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Optical density (a)	0.196	0.282	0.370	0.439	0.456	0.490	0.502	0.500	0.495	
Mean optical density for 100 per cent hemolysis (b)							0.499			
Per cent hemolysis	39.0	56.5	74.0	88.0	91.5	98.0				
($c = \frac{100a}{b}$)*										

*Calculated to the nearest 0.5 per cent.

The volume of 1:100 complement required for 50 per cent hemolysis is then determined by a precise graphic method which avoids construction of the hemolytic curve.⁴ Other methods employing the construction of this curve may be used with excellent results.^{1c, 2} Given x , the volume of 1:100 complement required for 50 per cent hemolysis, y , the dilution of stock 1:20 complement containing 1 unit in 0.4 c.c. is determined according to the relations

$$(1) \quad \frac{y'}{100} = \frac{0.4}{x} \quad \text{and} \quad (2) \quad y = \frac{y'}{20}$$

where y' is the dilution of undiluted complement containing one unit per 0.4 cubic centimeter. Substituting in Equation 2 the value of y' derived from Equation 1, and simplifying, gives

$$(3) \quad y = \frac{2}{x}$$

If, as in the titration of Table II, 0.332 c.c.* of 1:100 complement is the unit,

$$y = \frac{2}{0.332} = 6.024$$

and 1 unit complement for use in the amboceptor titration would be prepared by adding 5.02 c.c. of salt solution to 1.0 c.c. of stock 1:20 complement.

Technique of the Amboceptor Titration.—The amboceptor titration is carried out as illustrated in Table III. The reagents are added accurately in the

*The graphic method⁴ in this instance gives 0.166 c.c. of 1:100 complement as the unit for the conditions of the diagnostic tests which employ unit volumes (V) of reagents. Since the amboceptor titration employs the double volumes (2V) required for spectrophotometric determinations, twice 0.166, or 0.332 c.c. of 1:100 complement, represent the unit under the latter conditions (see Spectrophotometer).

order given, and the contents of each tube are thoroughly mixed. Thirty minutes in the water bath at 37° C. are allowed for hemolysis, after which the tubes are spun at 2,000 r.p.m. for ten minutes. The optical densities of their supernates are determined spectrophotometrically. Tube 10 of the completed complement titration (Table II) is re-used to set the instrument at "0" optical density, and the predetermined mean optical density of Tubes 7 to 9 (Table II) serves again as the standard of reference for 100 per cent hemolysis. The percentages of hemolysis in Tubes 1 to 7 are calculated as for the complement titration. The results of a sample determination are given in Table III.

TABLE III. AMBOCEPTOR TITRATION

	TUBE						
	1	2	3	4	5	6	7
	DILUTION OF AMBOCEPTOR						
	1:500	1:666	1:1000	1:1333	1:2000	1:2666	1:4000
1 unit complement (c.e.)	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Salt solution (c.e.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Sensitized cells (c.e.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Optical density (<i>a</i>)	0.274	0.259	0.251	0.211	0.146	0.075	0.026
Per cent hemolysis ($c = \frac{100a}{b}$)*	55	52	50	42	29	15	5
Units of complement re- quired for 50 per cent hemolysis (from Fig. 1, <i>A</i>)	0.97	0.99	1.00	1.05	1.15	1.30	1.56

*Calculated to the nearest 0.5 per cent.

If the 1 unit complement used in this titration has been correctly prepared, 0.4 c.e. should yield 50 per cent hemolysis of the cells sensitized with 1:500 amboceptor. Deviations from this percentage, while they do not invalidate the results of the amboceptor titration, should not exceed the range of 45 to 55 per cent hemolysis.

Determination of the Optimal Concentration of Amboceptor.—The number of complement units that would be required for 50 per cent hemolysis with the different dilutions of amboceptor is then determined from the percentages of hemolysis obtained with one 50 per cent unit in the titration. This determination is made by reference to the curve of Fig. 1, *A*, the construction of which is described in the next paragraph. Proceeding with Tube 1 of the titration and referring to Fig. 1, *A*, it is observed that if 55 per cent hemolysis is obtained with 1 unit of complement, 0.97 unit would be required for 50 per cent hemolysis. Values obtained in this way for Tubes 1 to 7 are included in Table III. They are plotted on cross-section paper, against the corresponding concentrations of amboceptor, and a smooth curve is fitted to the coordinate points. The method, as applied to the results of Table III, is illustrated in Fig. 1, *B*. It will be noted that coordinate points representing concentrations of amboceptor from 0.0004 to 0.0008 c.e. per 0.4 c.e. of the diluted reagent fall, for all practical purposes, on a straight line, while points representing lower concentrations deviate from

linearity. It has been our practice to select as optimal the lowest concentration of amboceptor falling on the apparently linear portion of the curve. In the present instance, such an optimum would be represented by a concentration of 0.0004 c.c. per 0.4 c.c. which corresponds with the 1:1,000 dilution. The dilution so selected should not cause agglutination of 2 per cent sheep's cells under the conditions in which these reagents are used in complement fixation tests. The optimal dilution need be redetermined only under conditions suggesting a deterioration of the amboceptor.

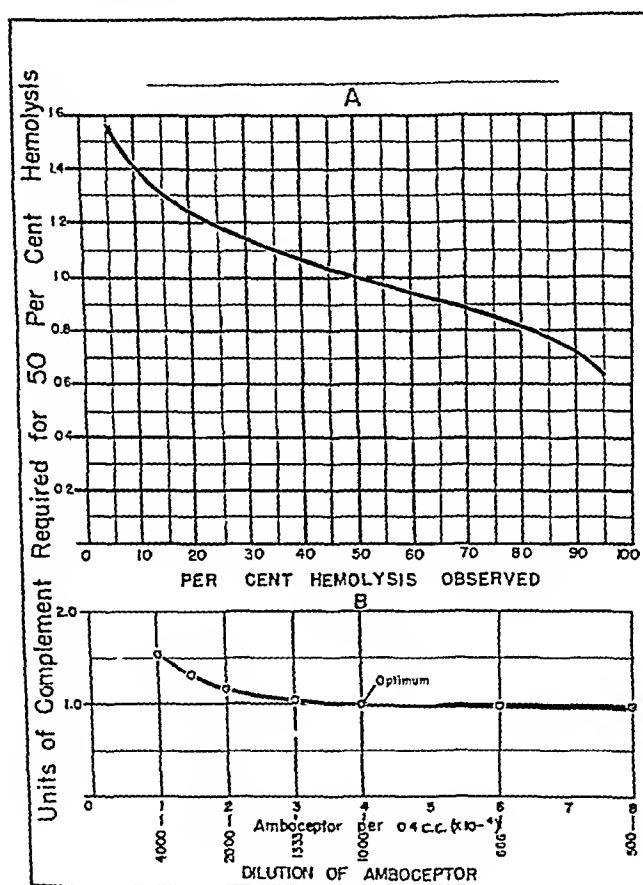


Fig. 1.

Construction of the Curve of Fig. 1, A.—The construction of this curve is based upon the observation that the curve of hemolysis is constant under given experimental conditions and may be expressed by the von Krogh alternation formula.⁷ The application of this formula to the standard conditions of the complement titration at this laboratory has been described, and volume factors have been derived which define the 50 per cent unit of complement in terms of the quantities of the reagent yielding other degrees of partial hemolysis.⁴ These factors are given in Table IV; plotted on arithmetic coordinates against the corresponding percentages of hemolysis, they yield the curve of Fig. 1, A.

TABLE IV. VOLUME FACTORS FOR CONSTRUCTION OF CURVE OF FIG. 1, A

PER CENT HEMOLYSIS OBSERVED WITH ONE UNIT OF COMPLEMENT	UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS	PER CENT HEMOLYSIS OBSERVED WITH ONE UNIT OF COMPLEMENT	UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS
5	1.56	55	0.97
10	1.40	60	0.94
15	1.30	65	0.91
20	1.24	70	0.88
25	1.18	75	0.85
30	1.14	80	0.81
35	1.10	85	0.77
40	1.06	90	0.72
45	1.03	95	0.64

During the course of these studies, it was observed that as the concentration of amboceptor is reduced below the optimum there occurs a slow increase in slope of the hemolytic curve as expressed by the constant $1/n$ in von Krogh's formulation. While derivation of the volume factors corresponding to these alterations in the value of $1/n$ would make possible slightly more accurate estimations of the 50 per cent unit of complement in the range of amboceptor deficiency, the observed error in the immediate range of the optimum is so slight as not to interfere with the selection of an optimal concentration of amboceptor by the present method. Moreover, the practical use of factors appropriate to varying degrees of amboceptor deficiency would presume knowledge of the optimum, which is itself the object of the determination, and they could accordingly not be applied *ab initio* in the standardization of an unknown antiserum.

SUMMARY AND CONCLUSIONS

A simplified technique has been developed for use in determining the optimal concentration of amboceptor for use in complement fixation tests. The method obviates both the preparation of color standards for measuring percentage of hemolysis and the construction of the hemolytic curve in determining the 50 per cent unit of complement. The percentage of hemolysis observed with one 50 per cent unit of complement in the presence of different concentrations of amboceptor is determined spectrophotometrically. The number of complement units required for 50 per cent hemolysis in each instance is estimated simply by reference to a standard curve which is represented graphically. The abbreviated method has been found to yield accurate and reproducible results.

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BOOK REVIEWS

Experimental Hypertension. Vol. 3. Edited by Roy Waldo Miner. Results of a conference on this subject held by the Section of Biology of the New York Academy of Sciences, Feb. 9 and 10, 1945, New York City. Special publication of the New York Academy of Sciences, New York, 1946. Price, \$3.75. Cloth, with 180 pages.

This publication consists of nine major papers presented at a conference on experimental hypertension by nineteen participants and covering as many viewpoints on the problem. In the Preface, Irvine H. Page states: "Investigation in the medical sciences often passes through several strata of development: the first, awareness that a problem exists, but widespread indifference to it; the second, attempted formulation of the problem along with much speculation; the third, widespread intellectual and emotional disagreement among those concerned with putting the problem to experimental test; fourth and last, agreement about the fundamental nature of the mechanism, application of the new knowledge to the care of patients, and commercialization. The testimony of the conference will leave little doubt that the third phase is beginning. It is a healthy state and one rich with promise."

The "intellectual and emotional disagreement" is summarized at the end of the book by William Goldring. There was a division of opinion on almost all points discussed at the conference. Such diversity of views does not make for easy reading, when presented in book form, in spite of denoting a "healthy state."

Although the conference was concerned with experimental hypertension, most of the papers considered various aspects of hypertension induced by interference with renal blood supply, with little discussion of hypertension induced by interference with various nervous pathways or by other means. Furthermore, it was naturally impossible to confine the discussion to experimental hypertension, *per se*, to the exclusion of arterial hypertension in man. However, most of the participants were careful to avoid applying the results of experimental work to human beings. Thus the book opens with a brilliant introductory lecture by Goldblatt in part of which the similarity of experimental renal hypertension and arterial hypertension in man is upheld. The book closes with a statement by Goldring, Chasis, and Smith, in which the opposite viewpoint is taken, that is, that the diminished renal blood flow which accompanies essential hypertension in man is merely a *secondary* effect "resulting from the action of a humoral pressor agent of unknown origin." Between these two positions the rest of the conference carefully avoided any commitment.

The various papers are well presented in a form suitable for publication in a journal. However, the wide divergence of views on a subject which invades so many fields gives the reader a sense of discontinuity. This is further amplified by the publication of full discussions of each paper.

As a reference book for workers in this specialized field, this publication presents the later ideas and summarizes the earlier work done by most of the groups who have contributed to our knowledge of experimental hypertension. However, it is doubtful whether this collection of papers would interest the casual reader, being just what it is meant to be—publication of a symposium on a highly specialized subject which is just beginning to open up and is therefore still confused.

THE CELLULAR MECHANISM OF RECOVERY AFTER TREATMENT WITH PENICILLIN

I. SUBACUTE BACTERIAL ENDOCARDITIS

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THE efficacy of a therapeutic agent may be evaluated in a number of different ways. The usual method is to observe the effect on the clinical course of the disease and to determine the per cent of patients who develop sequelae and the mortality rate with and without the agent. In this paper a little used but exceedingly valuable method of evaluation will be presented—the study of tissues from patients who have been treated with some therapeutic agent and subsequently die of the primary disease or of a complication.

This approach has the advantage of demonstrating the cellular mechanism by which a therapeutic agent facilitates healing. In this manner more may be learned of the nature of the disease, how progress of it is halted, and how repair is accomplished. Then the physician is in a position to employ the therapeutic agent more effectively or to reinforce the specific treatment with other agents.

BASIS OF STUDY

My interest in the process of healing after treatment with penicillin which led to the following investigation was first aroused in January, 1943. At that time the Department of Pathology at the Washington University School of Medicine performed an autopsy on a young woman, 33 years of age, who presumably had an induced abortion. On admission to the Barnes Hospital on Dec. 26, 1942, the patient stated that she had had prolonged vaginal bleeding which started a few days before her expected menstrual flow on December 19. Twenty-four hours before admission she experienced a severe shaking chill and thereafter had a fever. To summarize briefly, the physical signs and the course in the hospital were those of a staphylococcal septicemia with endocarditis. Cultures of the blood, sputum, urine, and uterine canal were positive for a hemolytic *Staphylococcus aureus*. The patient was given penicillin for five days for a total dose of 320,000 units. The blood culture became negative on the second day of treatment and remained so until death on Jan. 2, 1943, seven days after entry and five days after penicillin was first given.

The immediate cause of death was cerebral hemorrhage, probably from rupture of a mycotic aneurysm or erosion of an artery by an abscess, although neither of these could be demonstrated.

From the Department of Pathology, Washington University School of Medicine.

This investigation has been carried out under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Washington University.

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The histologic structure of the cervix, endometrium, a small bit of retained placenta, ovary with a corpus luteum, breast, and pituitary indicated that the woman had been pregnant for approximately three months and that an interruption of the pregnancy occurred about ten to twelve days before death and was followed by an infection. The lesions of greatest interest were in the heart and the kidneys.

Three vegetations on the thickened mitral valve were not unusual in gross appearance. Microscopically they were not typical of acute bacterial endocarditis. The most conspicuous difference from the characteristic appearance was the absence of a layer of bacteria and fibrin on the surface. A culture showed a few staphylococci, and in sections stained for bacteria a few gram-positive cocci were observed.

The kidneys were enlarged, and over the surface were numerous small (1 to 2 mm. in diameter), depressed, red foci. The foci were collections of lymphocytes, plasma cells, and a few leucocytes with minimal necrosis.

Although there are other possible explanations of these observations on the heart and kidneys, it seemed at that time that both might be considered as healing or abortive variants of staphylococcal pyemia.

PLAN OF STUDY

Because of the interest this case aroused, thereafter the autopsies on patients treated with penicillin at the Barnes Hospital were studied a little more than was required by routine demands. It became apparent that a satisfactory study could not be made on the limited material of one hospital. Accordingly, an application was made to the Committee on Medical Research of the Office of Scientific Research and Development. The suggested investigation contemplated collection of histories, autopsy protocols, and tissue from all hospitals in which penicillin was used in 1943 and 1944, this investigation to be conducted under the auspices of the Committee. The proposal was authorized, and in the fall of 1944 the materials for study were secured in visits to twelve hospitals and schools, the staff of which were most cooperative.

Over 100 cases were available for study in four categories, namely, subacute bacterial endocarditis, pyemia, pneumonia, and meningitis; of these, the first, subacute bacterial endocarditis, illustrates most of the general principles and will be discussed in this paper. No review of the literature is included but only personal observations, many of which have been made previously by others.

As a control, eight cases from the files of the Department of Pathology were studied. These patients died between 1933 and 1935 and received no specific therapy.

THE NATURE OF THE VEGETATION IN ENDOCARDITIS

As the study proceeded it became increasingly clear that the observations could not be explained if the usually accepted idea of the nature of a vegetative endocarditis were accepted. In most textbooks the terms vegetation and thrombus are used interchangeably, and it is stated or implied that the vegetation is a thrombus.

In a perfectly formed vegetation of bacterial endocarditis, acute or sub-acute, there are three definite layers: a large central mass of necrotic, relatively acellular, fibrillated debris; a more peripheral layer of bacterial colonies; and a most peripheral layer of delicate fibrillary acellular material. There are slight differences in layers 1 and 3, but superficially they appear to be similar. If a vegetation is stained for elastic fibrils or for collagen, fragments of these are seen in layer 1 but not in layer 3. Further, stains for fibrin show a network in both, but in layer 1 the threads of fibrin constitute a small part of the total mass.

Since elastic and collagen fibrils are observed regularly in the central core of the vegetation, which constitutes three-quarters to seven-eighths of the total, it logically follows that this part is necrotic tissue and not thrombus. Careful study of the pattern of the fibrillary structure further confirms this. The whorls are identical with those present in the valve of chronic endocarditis.

Discussion of the implications of this conclusion on the pathogenesis and postulated growth of vegetations will be postponed until presentation of the observations on healing of subacute bacterial endocarditis.

EFFECT OF TREATMENT WITH PENICILLIN ON SUBACUTE BACTERIAL ENDOCARDITIS

From the twenty-two cases studied, five have been selected which illustrate the essential features

CASE 1.—Barnes Hospital Autopsy No. 11,617. A 56 year old white woman was admitted to the hospital on March 14, complaining of the following symptoms: exertional dyspnea and pain in the cardiac region since childhood; weakness, loss of weight, and chills and fever for six months; and abdominal pain and swelling of the legs for two months.

There were petechiae in the conjunctivae and on the fingers and toes. The heart was slightly enlarged. The liver and spleen were palpable and tender. Blood cultures showed *Actinomyces bovis* and alpha hemolytic streptococci. Beginning on March 15, the patient was given 40,000 units of penicillin intramuscularly every two hours for four days without benefit. Sulfadiazine was then given, and levels of 10 to 15 mg. per cent were obtained. On March 23 penicillin was begun again at 40,000 units every two hours and increased to 80,000 units every two hours on March 26. Many embolic phenomena developed and cardiac failure increased. The patient died March 30. Penicillin was last given one hour before death, and the total dose was 6,720,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral and tricuspid valves; subacute bacterial endocarditis of the tricuspid and mitral valves and posterior wall of the left atrium; infarcts of the heart, spleen, liver, and kidneys; and focal embolic glomerulonephritis.

A section of the mitral valve (Fig. 1) shows two vegetations, one sessile mass (*A*) near the line of closure and a larger pedunculated vegetation (*B*) nearer the base. In Fig. 2 (designated as area 2 in Fig. 1) is shown the entire thickness of the pedunculated vegetation. There are thin layers of tissue on each side enclosing a necrotic centrum. The granulation tissue extends outward on the surface to a point just short of the large bacterial masses (Fig. 3, designated as area 3 in Fig. 1). A higher power view of the superficial tissue and deeper necrosis of area 2 is given in Fig. 4. The junction of the tissue with the necrotic bulbous end of the vegetation is depicted in Fig. 5.

The appearance of Figs. 1 to 5 may be explained as a proliferation of tissue through the superficial layer of fibrin and bacteria. As the capillary vessels, fibroblasts, and leukocytes advance, the bacterial colonies are destroyed. In this vegetation the process has extended about two thirds of the way from the base to the apex of the vegetation.

Fig. 2.

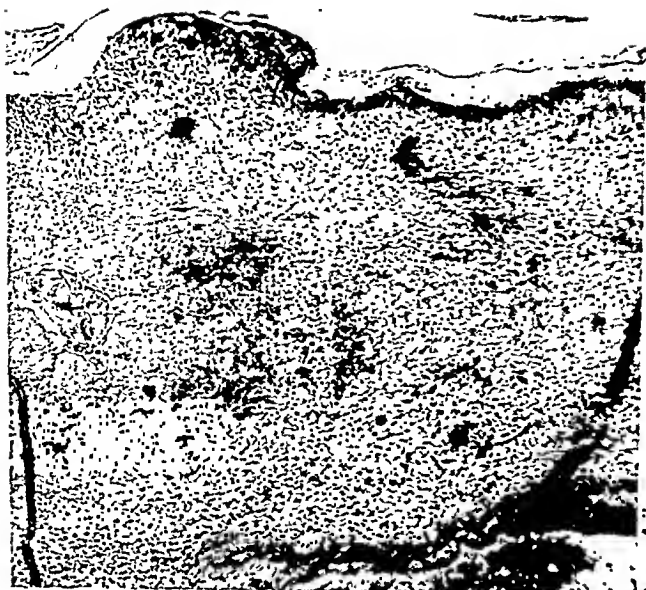


Fig. 1.



Fig. 3.

Fig. 1.—(Case 1.) Two vegetations of subacute bacterial endocarditis on the mitral valve, one pedunculated and one sessile.

Fig. 2.—(Case 1.) The stalk of the pedunculated vegetation from area 2, marked on Fig. 1. Note the tissue in the superficial parts on both sides and the central necrotic core.

Fig. 3.—(Case 1.) Higher power view of area 3, marked on Fig. 1. The tissue on the left is approaching the bacterial colonies on the right. Note that bacteria have disappeared from the part of the vegetation already organized.

Fig. 4.



Fig. 5.

Fig. 4—(Case 1) Higher power photomicrograph of area δ in Fig. 1. The superficial part of the vegetation is composed of a loose fibroblastic tissue with a distinct line of demarcation from the underlying necrotic core.

Fig. 5—(Case 1) Appearance of the more distal extension of the tissue in Fig. 3. As in Fig. 4 there is a sharp demarcation between the tissue and the necrotic core below.

In some instances organization may also proceed from the entire base of the vegetation. In the sessile vegetation *B*, there are successive layers of dense connective tissue, granulation tissue, lacunae filled with leucocytes, and bacterial colonies embedded in a granular debris (Fig. 6, designated *c* in Fig. 1). The lacunae (Fig. 7) probably represent former bacterial colonies which have been phagocytized and destroyed.

CASE 2.—Barnes Hospital Autopsy No. 11,779. A 55-year-old white man was admitted to the hospital on May 8, 1915. Five years previously he had a left-sided hemiplegia, with almost complete recovery except for general weakness until six weeks previously when he began to notice swelling of the feet and ankles and an increasing number of petechiae. More recently he had difficulty in speaking clearly.

On May 14 a blood culture showed alpha hemolytic streptococci. Penicillin was started May 17, 40,000 units every two hours intramuscularly for fourteen days. The blood cultures remained positive. On June 1 penicillin was increased to 50,000 units every hour. The organism was then found to grow in concentrations of 20 units of penicillin per cubic centimeter. Penicillin was stopped on June 5 and begun again, 50,000 units every hour, on June 12. On June 17 test injections of 100,000 units were given intravenously every two hours and blood levels taken. Five minutes after injection the level was about 16 units per cubic centimeter. On June 18, 50,000 units every hour intramuscularly were begun again and continued until June 20, for a total of 22,610,000 units.

At that time the patient refused further injections of penicillin and signed himself out of the hospital, under protest, on June 28. He was readmitted July 22; the edema was worse, the petechiae were more abundant, there were small pustules on both hands, and he complained of shortness of breath. No chemotherapy was attempted because of evidence of extreme renal and hepatic damage. The patient developed pulmonary edema on July 31 and died on Aug. 1, 1945.

The anatomic diagnosis included: chronic endocarditis of the mitral valve; subacute bacterial endocarditis of the mitral and aortic valves and wall of the left atrium; subacute bacterial endarteritis of the ascending aorta; recent and healed infarcts of the spleen and kidneys; focal encephalomalacia of the occipital lobes and left cerebellum; focal embolic glomerulonephritis; and multiple abscesses of the myocardium.

Sections from the vegetation on the mitral valve show well the process of phagocytosis of bacteria, destruction of the colonies, and formation of lacunae. In Fig. 8 part of a colony has been destroyed and the remainder broken into smaller fragments. A large lacuna with only a small mass of bacteria at one side is depicted in Fig. 9. As the colonies are destroyed, large numbers of bacteria are seen in phagocytes (Fig. 10). The same phenomenon is present in the mural vegetations of the left atrium in this heart (Fig. 11).

The observations illustrated in Figs. 6 and 7 from Case 1 and Figs. 8 to 11 from Case 2 may be explained as phagocytosis of the bacterial colonies when living tissue reaches the proximity of the bacteria. The end result is a lacuna surrounded by necrotic vegetation or tissue.

CASE 3.—Barnes Hospital Autopsy No. 11,103. A 68-year-old white woman was admitted to the hospital on January 27, complaining of fever and malaise of two and a half months' duration. The blood culture showed *Streptococcus viridans*. On February 8 the patient was transferred to another hospital and after three weeks with no improvement returned home. On March 15 the patient was readmitted to Barnes Hospital for penicillin therapy. She was disoriented and unable to speak or turn her body. There were many petechiae on the conjunctivae, face, trunk, and extremities, and the feet were swollen. The blood culture showed *Str. viridans*. Penicillin was begun on March 17, 25,000 units every two hours intramuscularly. On March 20 the blood culture was negative, but the patient failed to improve and died on March 23. Penicillin was last given five hours before death, and the total dose was 1,700,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral valve; subacute bacterial endocarditis of the mitral valve; partially healed infarcts of the spleen and kidneys; focal embolic glomerulonephritis; and subarachnoid hemorrhage.

Fig. 6.

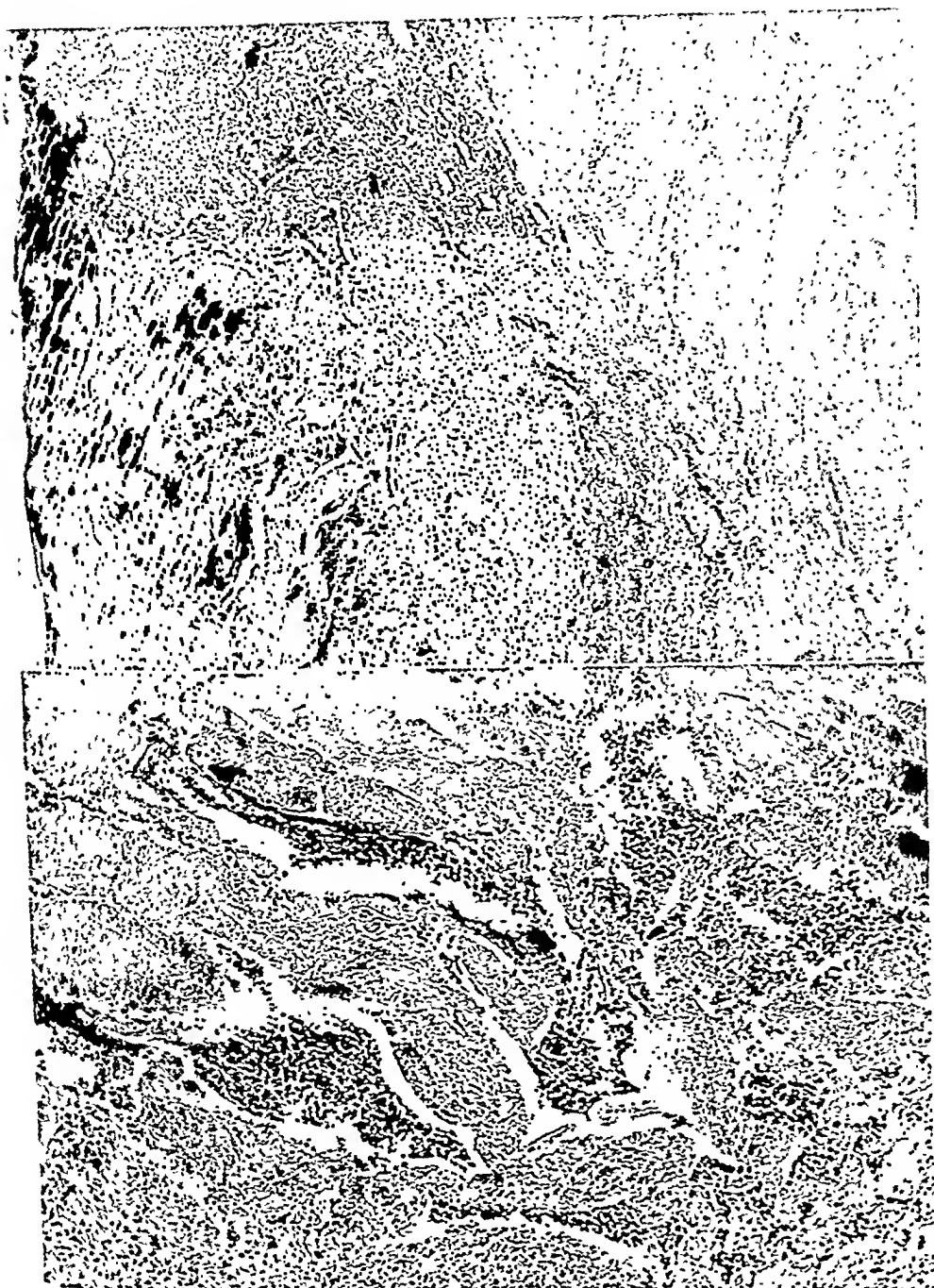


Fig. 7.

Fig. 6.—(Case 1.) Low power photomicrograph of the sessile vegetation designated as area 6 in Fig. 1. Superficially to the left there are fibrin and bacterial colonies. In the right there are many leucocytes and granulation tissue. On the extreme right is the fibrotic valve.

Fig. 7.—(Case 1.) A higher power photomicrograph of the central area of Fig. 6. Note the clefts filled with polymorphonuclear leucocytes representing destroyed bacterial colonies.

There is a large partially organized vegetation on the mitral valve (Fig. 12). In the area outlined there is a sharp line of demarcation between the necrotic part and the proliferating fibroblasts. This is taken to mean that the necrotic component of the vegetation organizes slowly or not at all. This conclusion is supported by the better known examples from general pathology of slow organization of the necrotic center of caseous tubercles and gummas.

CASE 4.—University of Minnesota Autopsy No. 41-999. A 37-year-old white woman was admitted to the hospital on May 5, with a three months' history of chills, fever, and weakness of the left side of her face, the right arm, and the right leg. Despite previous treatment with large amounts of sulfanilamide in another hospital, the blood culture showed *Str. viridans*.

The heart was enlarged to the left, the liver and spleen were palpable, and there was a large umbilical hernia. There were many petechiae on both conjunctivae. Penicillin was started May 6 and given intravenously, 100,000 units every twelve hours. The patient improved, and the blood culture showed no streptococci after twenty-four hours. New petechiae appeared in the conjunctivae, however, and on May 30 the patient suddenly developed sharp abdominal pain localized in the umbilical region. An exploratory laparotomy, performed because of the possibility of a strangulated abdominal hernia, revealed a generalized peritonitis. The patient died on the operating table. Penicillin had been continued to the day of death, the total dose being 4,200,000 units.

The anatomic diagnosis included: chronic endocarditis of the mitral valves; subacute bacterial endocarditis of the mitral valve; suppurated infarcts of the spleen with rupture and peritonitis; focal embolic glomerulonephritis; bilateral pulmonary atelectasis; and acute arteritis of the hepatic artery with thrombosis.

This case is used to illustrate advanced healing of a small vegetation. In Fig. 14 there are three distinct strata: a central calcified core, a middle layer of hyalinized and necrotic tissue, and a superficial layer of loose proliferating fibrous tissue.

The appearance in Fig. 14 could be compared to that in Fig. 2, if the central part of the latter were to become compacted and the centrum to calcify.

Fig. 15, from a case not given in detail (University of Minnesota 41-1468), shows calcification of an individual bacterial colony.

CASE 5.—Barnes Hospital Autopsy No. 11,358. A 46-year-old white man was admitted to the hospital on July 3, complaining of pain and edema in the left leg for one month, exertional dyspnea for three weeks, and a pruritic rash for three days. The patient had an attack of rheumatic fever at 21 years of age and another a year later.

The heart was enlarged and there was a harsh systolic murmur over the precordium. The liver was enlarged. On July 5 a blood culture showed hemolytic streptococci, the patient had a fever, and the spleen was palpable.

On July 15 penicillin was started, 20,000 units every two hours intramuscularly. After July 16, the next day, all blood cultures were negative and the temperature was normal. Penicillin was discontinued August 9, after a total of 6,080,000 units had been given in twenty-six days. The patient was discharged, improved, on August 23.

He was readmitted on August 30, complaining of increased dyspnea and edema of the ankles. On September 20 the patient had a sudden attack of pulmonary edema and died shortly afterward.

The anatomic diagnosis included chronic endocarditis of the aortic valve with calcification and aortic stenosis.

The aortic valve (Fig. 16) is greatly deformed with a thin base and a large bulbous calcified edge. The calcified masses are surrounded by a loose fibroblastic tissue infiltrated with lymphocytes and large mononuclear cells (Fig. 17). In other regions, especially near the surface, there are endothelial-lined spaces (Fig. 18), variable in size and outline but corresponding in general architectural pattern to the incision left by destruction of bacteria shown in Fig. 7. The surface is lined by endothelium, and all evidence of a vegetation is absent.

Fig. 8.

Fig. 9.



Fig. 10.

Fig. 11.

Fig. 8.—(Case 2.) Partial organization of a bacterial colony.

Fig. 9.—(Case 2.) Clear area in the superficial part of the vegetation partially filled with leucocytes and with the remnants of a bacterial colony.

Fig. 10.—(Case 2.) Active phagocytosis of bacteria.

Fig. 11.—(Case 2.) Invasion of the bacterial colonies from below in a mural vegetation. Lesion on the wall of the left auricle.

Fig. 12.



Fig. 13.

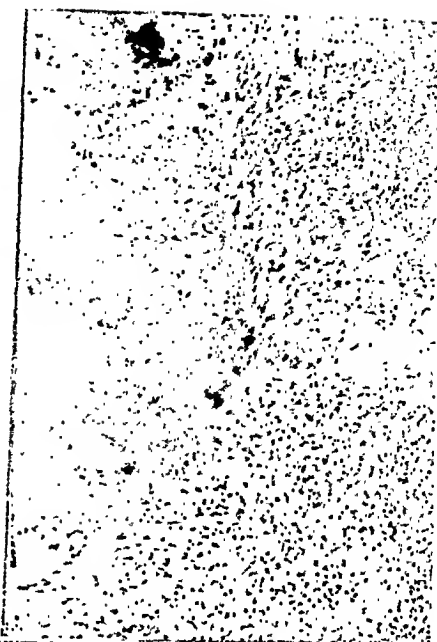


Fig. 14.



Fig. 15.

Fig. 12.—(Case 3.) Partially organized vegetation on the mitral valve.

Fig. 13.—(Case 3.) From the designated area on Fig. 12 to show the sharp line of demarcation between the necrotic part of the vegetation and the organizing part.

Fig. 14.—(Case 4.) Calcification of the central part of a completely organized vegetation surrounded by a layer of cartilaginous connective tissue and covered on the surface by a loose fibroblastic tissue.

Fig. 15.—(Case not given in detail.) Calcification of a bacterial colony.

Fig. 16.



Fig. 17.



Fig. 18.

Fig. 16.—(Case 5.) Calcified nodules of the aortic valve in a patient who died two months after apparent cure of subacute bacterial endocarditis.

Fig. 17.—(Case 5.) A calcified nodule surrounded by loose connective tissue.

Fig. 18.—(Case 5.) Large endothelial lined spaces in the superficial part of the aortic valve shown in Fig. 16.

The observations of this valve with advanced healing serve to confirm the conclusions reached from study of the earlier stages of healing seen in the other cases. The vegetation organizes or calcifies, and the bacteria are destroyed, the spaces occupied by them becoming endothelized.

HEALING IN SUBACUTE BACTERIAL ENDOCARDITIS

On the basis of these observations, healing in subacute bacterial endocarditis may be arranged as five processes: covering of the exposed surface of the vegetation with fibrous tissue, invasion of the layer of colonies and phagocytosis of bacteria, calcification of bacterial colonies, hyalination and calcification of the central core of the vegetation, and endothelization of the spaces and clefts in the vegetation.

In order to understand the first process, covering of the exposed surface of the vegetation with fibrous tissue, it is necessary to return to the discussion of the nature of the vegetation in bacterial endocarditis. To recapitulate, it is composed of a central core of necrotic tissue, a layer of bacterial colonies embedded in fibrin, and a superficial thin layer of fibrin. The necrotic core is similar to other foci of dead tissue such as the caseous nodule of tuberculosis. There is no network of fibrin on which an invading granulation tissue may form. Hence, it undergoes organization slowly or not at all. On the other hand, the superficial layer is fibrin and is readily and quickly replaced by fibrous tissue. The limiting factor is the state of the fibrous tissue at the edge of the vegetation. If this region is well vascularized and if the fibrous tissue is not too collagenous and hyalinized, proliferation will proceed from some or all directions. In time the central core will be completely covered. Concurrently, the fibrous tissue about the base of the vegetation is active in isolating the necrotic mass and invading it along the few septa and at places where a network of fibrin exists. The end result is a central necrotic mass completely surrounded by tissue.

The second process, invasion of the layer of colonies and phagocytosis of the bacteria, is a phenomenon seen in all bacterial diseases. In the absence of a specific chemical or biologic agent which will kill bacteria, the only method for disposal of the bacteria is to bring active phagocytes to the infected focus. In subacute bacterial endocarditis of a valve, this is not possible until the first process, covering of the surface with tissue, has taken place. A possible alternate is organization of the necrotic center, but it has been pointed out that this does not occur except in small superficial vegetations. In other words, leucocytes cannot migrate through the necrotic core, nor will they go through the superficial layer of fibrin from the blood in the cavities of the heart in sufficient numbers. That the latter is attempted is suggested by the alignment of leucocytes on the surface of the vegetation. As fibrinoblasts and capillaries replace the peripheral layer of fibrin, the adjacent bacterial colonies are broken apart and the bacteria are phagocytized and destroyed, at least in large part.

For reasons not apparent from the histologic study, some bacterial colonies undergo calcification, the third process. Perhaps the bacteria die and attract calcium salts in the same manner that necrotic tissue does.

The fourth process, hyalination and calcification of the necrotic part of the vegetation, is an undesirable feature of healing. As previously pointed out, necrotic tissue is not ideal soil for organization. There is no pre-existent framework of fibrin for the fibrinoblasts and capillaries. Therefore there is encapsulation with hyalination and calcification. This process does not involve any significant degree of contraction or decrease in size. Hence the end result is a greatly deformed nodular valve, similar to that of calcific stenosis.

As the bacterial colonies are destroyed and as the necrotic core becomes lobulated, clefts and spaces appear in the vegetation. Endothelial cells from the surface or from sprouting capillaries grow into and line the spaces, the fifth process.

In summary, healing in subacute bacterial endocarditis is essentially the conversion of a vegetative endocarditis into a chronic endocarditis characterized by superficial vascularized connective tissue and central nodular calcification. Only time will tell whether or not the latter disease is functionally significant. Reports so far suggest that some patients develop cardiac failure as a result of the calcific stenosis, as did one of the patients reported on in this paper.

TREATMENT OF SUBACUTE BACTERIAL ENDOCARDITIS

Comparison of the observations in those patients treated with penicillin and in those patients who received no specific therapy indicates that there is some evidence of healing in all, regardless of the form of treatment. However, more advanced healing is observed after treatment with penicillin.

Logically there are four possible procedures by which healing might be facilitated: (1) Control the further growth of bacteria; (2) bring phagocytes to the bacteria; (3) promote the growth of granulation tissue; and (4) aid in the final disposal of necrotic tissue.

Clearly, penicillin is effective in the first procedure, control of further growth of bacteria. This inhibition, regardless of how produced, will be reflected in greater phagocytosis and proliferation of tissue, since both are unfavorably influenced by active multiplication of bacteria. The sooner the inflammation is brought to a standstill the more effective the final disposal of the necrotic debris. Hence, penicillin by an action of simple bacteriostasis exerts an effect on all aspects of healing. Therefore, in the sections from patients treated with penicillin, there is more extensive and advanced healing than in those not so treated.

The dose and duration of treatment are largely outside the domain of the pathologic anatomist. However, it is suggested that a blood level effective bacteriostatically on the organism of the patient be maintained for the period it takes for the layers of bacterial colonies and fibrin to be replaced by tissue. The actual range is probably 200,000 to 1,000,000 units per day in not less than six divided doses intramuscularly for three to six months.

This study was originally undertaken to determine whether or not better forms of treatment could be developed. Penicillin has a direct effect on only one of the four possible modes of attack. Perhaps when really effective agents become known, it may be possible to accelerate the phagocytosis.

Some of the factors determining the healing of wounds are already understood. The vegetation is a wound which must be healed in part by fibrosis. Among other things, an adequate supply of protein and vitamin C is necessary for rapid proliferation of normal fibroblasts. The most careful attention should be given to the nutritional status of the patient with subacute bacterial endocarditis, particularly to the level of the plasma albumin and to the adequacy of available vitamin C.

If the vegetation is largely necrotic tissue, it follows that it does not grow by addition of fibrin on the surface. Hence, there is no basis for the use of an anticoagulant such as heparin to prevent a process which does not occur.

Whether or not it is feasible or desirable to accelerate calcification, will be left for others to decide. It has been reported that calcification of caseous tuberculous nodules in rabbits may be hastened by a high calcium diet and vitamin D. Perhaps a similar procedure initiated toward the end of active treatment would be worth while. Possibly further research may reveal a technique for disposal of the necrotic tissue which does not lead to calcification and deformation of the valve.

PATHOGENESIS OF SUBACUTE BACTERIAL ENDOCARDITIS

It is not possible to study the what, that is, the pathologic anatomy of a disease, for long and not wonder about the how and why.

How and why do people develop subacute bacterial endocarditis? Of the thousands who have rheumatic fever, only a small percentage have this complication. Of the thousands who have a tooth extracted or have an acute upper respiratory infection, only a very few develop an endocarditis. Even when these two states, lesions of rheumatic fever and streptococci, are present together, the known incidence cannot account for the onset of subacute bacterial endocarditis.

The basic part of all vegetations is the same, the necrotic core of a valve. This is true for the verrucae of rheumatic fever, the vegetation of nonbacterial thrombotic endocarditis, and for the lesions of bacterial endocarditis. Bacterial endocarditis differs from the others only in that there is a superficial layer of bacteria and fibrin. May it not be postulated, then, that bacterial endocarditis consists of two parts, a nonbacterial primary part and a bacterial secondary part?

It is well known that nonbacterial thrombotic endocarditis is a not uncommon observation at autopsy in those who have died of debilitating diseases. Perhaps it occurs and heals more frequently than is suspected. It might account for the fibrous thickening of valves and adhesions at the commissures of the aortic valves observed in 60 to 90 per cent of all hearts at autopsy.

It is therefore submitted as a working hypothesis that if a person has a nonbacterial endocarditis, either rheumatic or thrombotic, at a time when he also develops a bacteremia with *Str. viridans*, he or she may develop subacute bacterial endocarditis.

This would explain (1) the known association of lesions of rheumatic fever and subacute bacterial endocarditis in the same heart, (2) the frequency of

lesions of subacute bacterial endocarditis on previously damaged valves, and (3) the low over-all incidence of subacute bacterial endocarditis in relation to previously postulated causal factors.

SUMMARY

A comparison has been made of the lesions of the heart in subacute bacterial endocarditis in twenty-two patients treated with penicillin and in eight patients given no specific therapy.

Penicillin promotes healing in subacute bacterial endocarditis, but the basic processes of healing are not modified. These processes are: covering of the exposed surface of the vegetation with fibrous tissue, invasion of the layer of colonies and phagocytosis of bacteria, calcification of bacterial colonies, hyalinization and calcification of the central core of the vegetation, and endothelialization of the spaces and clefts in the vegetation.

In some patients, healing is accompanied by excessive calcification, and the result is a calcific stenosis of the valve.

It is postulated that the vegetative lesions of subacute bacterial endocarditis consist of two parts, a nonbacterial (rheumatic or thrombotic) primary part and a bacterial secondary part.

EFFECTIVENESS OF CONJUGATED FORMS OF FOLIC ACID IN THE TREATMENT OF TROPICAL SPRUE

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FOLIC acid (pteroylglutamic acid) occurs naturally not only as the free vitamin, but also in the form of various complexes or conjugates. Two of these complexes, both polyglutamates, have been isolated in pure form, one from yeast and one from a filtrate obtained from an aerobic fermentation of a diphtheroid type organism. The folic acid complex of yeast, often termed vitamin B₁₂ conjugate, was identified by Pfister and co-workers^{1,2} as pteroylhexaglutamylglutamic acid. The other naturally occurring complex, usually termed fermentation folic acid or fermentation *Lactobacillus casei* factor, was described by Hutchings, Stokstad, and their collaborators^{3,4} and was shown to be a triglutamate (pteroyldiglutamylglutamic acid).

The ability of these complexes of folic acid to affect the growth of those organisms unable to synthesize folic acid shows certain interesting differences. Thus, fermentation folic acid appears to be utilized effectively by *L. casei*, exerting an effect deviating from that of crystalline pteroylglutamic acid (PGA) only as the molecular weights differ; for *Streptococcus faecalis* (*Streptococcus lactis* R) the compound has only about one-fiftieth the activity of pteroylglutamic acid. The conjugate of yeast (heptaglutamate) is essentially unable to stimulate the growth of either microorganism. In animal species known to require folic acid (monkey, chick, rat), each of the conjugates exerts an effect essentially equivalent to its folic acid content, and it is probable that this activity results from these compounds serving as a source of free folic acid, split off in each case by the action of a group of enzymes widely distributed in tissues and generally termed conjugases. The possibility has not been excluded, however, that the conjugates may also be utilized as such.

The effectiveness of folic acid in the treatment of pernicious and other forms of macrocytic anemia, including sprue, led to the investigation of these complexes as examples of the types of compounds from which human subjects presumably must derive a considerable portion of their supply of folic acid.

It has already been reported by Welch and associates^{5,6} and by Bethell and co-workers⁷ that the conjugated folic acid of yeast is little or not at all utilized by patients with pernicious anemia, although normal human subjects demonstrated their ability to derive folic acid from the same lots of this heptaglutamate by a marked augmentation of the urinary excretion of the free vitamin following the oral or parenteral administration of the conjugate. It was suggested by both groups of workers that the folic acid deficiency char-

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acteristic of pernicious anemia might be due, at least in part, to the inability of such patients to obtain adequate amounts of the free vitamin from the naturally occurring complexes found in the diet and that as a result of this biochemical defect the supply of free folic acid is insufficient for the maintenance of normal hematopoiesis. It was suggested by Welch and associates^{5, 6} that liver factors have a more deep-seated action than that solely concerned with correcting a defect in the utilization of conjugates of folic acid, since such extracts have produced a striking reticulocytosis following an inadequate response to prolonged dosage with synthetic folic acid.

Another complication has been offered by the finding that some patients with pernicious anemia, of a type as yet indistinguishable from that previously described, are able to utilize concentrates of the yeast form of conjugated folic acid. Of such a type, Sharp⁸ has observed two examples and Castle,⁹ one: while one patient with pernicious anemia in remission induced by liver extract, but maintained for more than six months on folic acid without liver extract, has been observed by Heinle and associates to utilize the heptaglutamate, as evidenced by the urinary elimination, in the form of free *L. casei* factor, of a large proportion of the administered conjugate.

It is clear that some patients with pernicious anemia are unable to release folic acid from the preparations of heptaglutamate used,⁸ while in other patients this biochemical defect is not present. Conceivably, such differences in handling the conjugate might reflect etiologic differences in the disease or might represent quantitative differences in the degree of deficiency of intrinsic factor or of erythrocyte maturation factor. On the other hand, it is now known that one or more substances occur naturally (for example, in yeast) that are capable of competitively inhibiting some of the conjugases which release folic acid from pteroylhexaglutamylglutamic acid.[†] The possibility has not yet been excluded that the concentrates of vitamin B₁₂ conjugate used in patients with pernicious anemia, who are able to derive folic acid from them, differed from those which caused neither a clinical response nor the appearance of folic acid in the urine. Although one lot of material that produced no response, in small daily doses or in a single dose of 30 mg.,⁶ represented apparently pure, crystalline pteroylhexaglutamylglutamic acid, other studies were carried out with concentrates that probably contained inhibitors of conjugases.[‡] It is not inconceivable that one of the defects in patients with

*Eight patients of this type have been described in reports by investigators at Ann Arbor and Cleveland.

†Personal communication from Dr. John R. Totter, School of Medicine, University of Arkansas, Little Rock, Ark. The inhibitory substance used by Dr. Totter was supplied by Dr. Sarah Ratner and corresponds to the glutamic acid polypeptide of *p*-aminobenzoic acid (Ratner, S. Blanchard, M. and Green, D. E. Isolation of a Peptide of *p*-Aminobenzoic Acid From Yeast, *J. Biol. Chem.* 164: 691, 1946). It was found that the affinity from five to ten times as great as vitamin B₁₂ conjugate for the conjugase derived from chicken pancreas and rat liver, but the response was not always obtainable and the inhibitor appeared not to be identical with a material causing inhibition of a conjugase derived from hog kidney. Using a preparation generously supplied by Dr. Ratner, two of us (A. D. W. and E. M. N.) have found that extracts of human bone marrow, whose ability to release folic acid from pteroylhexaglutamylglutamic acid was measured at pH 4.5, are inhibited by the polyglutamate of *p*-aminobenzoic acid but quantitative data are not yet available.

‡In a personal communication Dr. J. J. Paffner has stated that the concentrates we employed were found by Dr. O. D. Bird to be essentially free of all inhibitor for hog kidney conjugase; however, information is not yet available concerning the presence of inhibitors (such as the glutamic acid polypeptide of *p*-aminobenzoic acid) of the conjugase found in human bone marrow.

pernicious anemia will be found to consist of a partial to complete inability to inactivate such inhibitors of conjugases; this possibility has been anticipated in previous papers.^{5, 6}

Spies¹⁰ has recently reported that fermentation folic acid, in a daily intramuscular dose of 3 mg., given during a period of eleven days, caused a submaximal reticulocyte response in a patient with pernicious anemia in relapse. Urinary excretion data were not presented. Although it has not been shown that treatment with liver extracts will restore the ability to release folic acid from its conjugates in patients previously unable to utilize such material, it is worth noting that the patient studied by Spies had received injections of liver extracts at some unstated time in 1946. It will be necessary and desirable to test the triglutamate in patients with pernicious anemia in severe relapse who have received no exogenous source of liver factors, at least for several months. However, the most recent findings, some of which are presented here, suggest that fermentation folic acid will not require liver extract factors to permit its utilization.

The supply of pteroyldiglutamylglutamic acid has been exceedingly limited and it has not been possible to obtain extensive information concerning its effect in tropical sprue. Similarly, the supply of the yeast form of conjugate has been restricted by the great difficulties attendant to isolation. In this report are described the striking clinical effect and hematologic responses, together with data on the urinary excretion of folic acid, obtained in two patients with tropical sprue. These patients were treated (by R. M. S. and R. M. S., Jr.) in the School of Tropical Medicine and University Hospital in San Juan, while the analytic studies of the urine were carried out in Cleveland (by A. D. W., R. W. H. and E. M. N.).*

CLINICAL OBSERVATIONS

Patient M. Q. (Unit history A-8757), a white, Puerto Rican man, 48 years of age, was admitted to the University Hospital on June 12, 1946, with the chief complaints of weakness, diarrhea, and weight loss (about forty pounds) during a period of about one year.

He stated that he was feeling well and that he had eaten what he considered to be a well-balanced diet until one year prior to admission, when he first noticed diarrhea. The diarrhea was described as watery, greenish-yellow, foamy, without mucus or blood, with from three to ten stools daily. With the onset of diarrhea, the patient developed flatulence and anorexia. Soon thereafter he noticed soreness of the mouth, with gradually increasing generalized weakness.

A few months after the onset of illness, a few injections of concentrated liver extract were given at irregular intervals and in inadequate doses. He received the last injection of liver extract two days before hospitalization and fourteen days before injections of fermentation folic acid were started.

On physical examination the patient was found to be in a cachectic condition, extremely pale, and obviously very weak and malnourished. A superficial, dry, scaly, brownish dermatosis was noticed over both ankles and wrists. The tongue was pale and fissured, and, although covered by papillae, these were very small and some of them over the tip and margins of the tongue were definitely inflamed. No aphthae were seen.

On admission, examination of the blood disclosed 1,200,000 erythrocytes per cubic millimeter; hemoglobin, 5.8 Gm. per 100 c.c.; leucocytes, 5,400 per cubic millimeter; mean cell

*The materials used in the studies were obtained through the courtesy of the Lederle Laboratories, Inc., Pearl River, N. Y. (fermentation folic acid) and of the Laboratories of Parke, Davis & Co., Detroit, Mich. (concentrate of vitamin B₁₂ conjugate).

volume, 160 μ ; the corrected sedimentation rate was 18 mm. per hour. Bone marrow aspiration revealed 25 per cent megaloblasts and 12 per cent normoblasts, with an erythrocytic series of 55 per cent and a granulocytic series of 37.8 per cent. Chemical findings in the blood were within normal limits, except for a serum protein level of 4 Gm. per 100 c.c., with a normal albumin-globulin ratio. Gastric analysis showed hypochlorhydria. Serologic and urinary findings were negative. Examination of the stool showed free fatty acids, soapy fats, and neutral fat (vide infra).

Laboratory Examinations.—Urine analysis revealed no abnormalities. Examination of stools disclosed hookworm ova. Analytic findings in blood were as follows: nonprotein nitrogen, 26.4 mg. per 100 c.c.; urea nitrogen, 12.7 mg. per 100 c.c.; sugar, 72.7 mg. per 100 c.c.; cholesterol, 108.6 mg. per 100 c.c.; phosphorus (inorganic), 2.7 mg. per 100 c.c.; calcium, 7.8 mg. per 100 c.c.; phosphatase, 4.7 Bodansky units; van den Bergh reaction, delayed; total serum protein, 4.0 Gm. per 100 c.c. (albumin, 2.6 Gm. per 100 c.c.; globulin, 1.4 Gm. per 100 c.c.); icterus index, 3.2 per cent; chlorides (as NaCl), 461.0 mg. per 100 c.c.

Gastric analysis after stimulation with histamine was as follows:

	FREE HYDROCHLORIC ACID (°)
Fasting	0
First	40
Second	26
Third	12
Fourth	0
Lactic acid, absent	

Biophotometric curve began at 26 and finished at 63 (normal, 55 to 100). Analysis of the dry solids (22 per cent) of a stool sample was as follows: total fat, 78.7 Gm. per 100 Gm.; free fatty acids, 65.7 Gm. per 100 Gm.; soaps, 0.9 Gm. per 100 Gm.; total split, 66.7 Gm. per 100 Gm.; neutral, 9.4 Gm. per 100 Gm.; unsaponified, 2.6 Gm. per 100 Gm.

Treatment.—The patient was placed on what we have referred to as a preliminary sprue diet, and this dietary regime, high in carbohydrates and very low in animal proteins, was maintained throughout the experiment. Twelve days after admission, when all laboratory examinations had been performed and various base lines established, daily intramuscular injections of fermentation folic acid were begun. The solution used was prepared by one of us (A. D. W.), in San Juan from a powder of 65 per cent purity. This material was dissolved in water and the pH adjusted to approximately 7.4 with the aid of sodium hydroxide. The solution was designed to contain, in each 2 c.c., approximately 3.0 mg. of potential folic acid. After autoclaving at 15 pounds pressure for fifteen minutes, a sample of the solution was withdrawn aseptically, hermetically sealed in a sterile capillary tube, and taken to Cleveland for analysis. Microbiologic assay (*L. casei* and *Str. faecalis*) before and after treatment with hog kidney conjugase¹¹ indicated that each daily dose (2 c.c.) of the solution contained approximately 4.9 mg. of fermentation folic acid, equivalent to about 3.1 mg. of pteroylglutamic acid. When treatment with fermentation folic acid was initiated, on June 24, 1946, the following hematologic observations were made: erythrocytes, 800,000 per cubic millimeter; hemoglobin, 4.2 Gm. per 100 c.c.; mean cell volume, 175 μ ; leucocytes, 2,300 per cubic millimeter; the reticulocytes, which originally were at an 8 per cent level, now composed 2 per cent of the circulating red cells. The patient was having from six to eight bowel movements daily and the body weight was only 96 pounds.

Three days after initiation of treatment with fermentation folic acid, the patient volunteered the statement that he felt better; the bowel movements had decreased to three daily; the number of megaloblasts in the sternal bone marrow was reduced to 1.2 per cent (from 25 per cent); the appetite had improved markedly and the patient experienced a sense of well-being. In spite of the inadequate diet, there occurred a definite improvement in the number and character of the stools. By the fourth day the stools were of normal consistency.

TABLE I. HEMATOLOGIC DATA OBTAINED IN PATIENT WITH SPRUE*

DATE	HEMOGLOBIN (GRAMS PER 100 C.C.)	ERYTHROCYTES (MILLIONS PER C.MM.)	LEUCOCYTES (THOUSANDS PER C.MM.)	RETICULO- CYTES (PER CENT)
6/24/46	4.2	0.80	2.3	2.0
6/26/46	4.8	0.82	1.7	5.2
6/27/46	4.8	0.81	1.1	6.0
6/28/46	4.0	0.80	1.0	18.0
7/ 1/46	5.4	1.21	2.1	12.4
7/ 2/46	6.6	1.20	3.6	17.8
7/ 3/46	5.8	1.28	3.7	12.8
7/ 4/46	7.2	1.59	3.5	15.8
7/ 5/46	6.8	1.75	2.2	11.0
7/ 6/46	--	--	--	11.4
7/ 8/46	7.2	1.73	4.3	9.6
7/ 9/46	7.6	1.83	3.8	9.4
7/10/46	8.1	2.09	4.9	6.8
7/12/46	8.4	2.39	5.6	11.2
7/16/46	10.5	2.60	3.0	11.4
7/24/46	10.1	3.03	5.3	3.2
7/29/46	11.4	3.46	4.7	3.8
8/ 5/46	12.8	3.52	4.0	0.3
8/12/46	12.0	3.66	5.1	0.3

*The patient (M. Q.) was given daily intramuscular injections of 4.9 mg. of fermentation folic acid (equivalent to 3.1 mg. of free folic acid), beginning June 24 and continuing through July 7, 1946 (14 injections). Additional therapy with liver extract (1 c.c. daily, containing 15 units) was begun on July 4 and was continued through Aug. 4, 1946.

On the fourth day of treatment the reticulocyte level had reached a peak of 18 per cent, and a level of about 18 per cent was maintained until the tenth day of treatment, July 4, when the daily administration of 1 c.c. of liver extract (15 units) was begun, in addition to the same dose of fermentation folic acid. On this day the erythrocyte count was 1,590,000 per cubic millimeter of blood and the hemoglobin level was 7.2 Gm. per 100 cubic centimeters. The number of leucocytes and of platelets had shown little change. The body weight had increased by six pounds.

Following the injections of liver extract, in addition to those of fermentation folic acid, the number of white blood cells and platelets increased, and there was a persistence of the reticulocytosis, a progressive increase in the number of erythrocytes and in the amount of hemoglobin, and a definite change toward complete recovery.

On July 16, twenty-two days after treatment was begun, the erythrocyte count was 2,600,000 per cubic millimeter; the hemoglobin level, 10.5 Gm. per 100 c.c.; mean cell volume, 141 μ ; leucocytes, 3,000 per cubic millimeter; and the reticulocyte level was 11.4 per cent. The body weight was 112 pounds (an increase of sixteen pounds).

Patient J. Q. (Unit history A-9216), a white man, 53 years of age, was admitted to the University Hospital on Sept. 5, 1946, with the chief complaint of generalized weakness during the past three months, accompanied by foamy diarrhea, a burning sensation of the tongue, flatulence, anorexia, and marked loss of weight. The stools were described as yellowish, abundant, fetid, either liquid or semisolid, with foam and mucus but no blood, and as occurring anywhere from twelve to eighteen times in twenty-four hours.

His past medical history was irrelevant, except for the fact that he had been on a poorly balanced diet for a number of years.

Physical examination revealed a short, poorly nourished, pale, cachectic, white man, who weighed only 68 pounds. The tongue was red and smooth, showing the typical appearance of an atrophic glossitis. No aphtha was found. The rest of the examination, including the neurologic, yielded essentially normal findings.

Röntgenologic study of the small intestine showed segmentation of the jejunum.

Laboratory Examinations.—The stools contained the ova of hookworm. The glucose tolerance curve was flat and showed an increase of only 15 mg. per 100 c.c. Analytic findings in blood were as follows: nonprotein nitrogen, 23.3 mg. per 100 c.c.; urea nitrogen,

13 mg. per 100 c.c.: cholesterol, 120 mg. per 100 c.c.: phosphorus (inorganic), 4 mg. per 100 c.c.: calcium, 8.6 mg. per 100 c.c.; phosphatase, 1.7 Bodansky units; van den Bergh reaction, delayed; total serum protein, 5.8 Gm. per 100 c.c. (albumin, 3.2 Gm. per 100 c.c.; globulin, 2.6 Gm. per 100 c.c.); ieterus index, 9.2 per cent.

Gastric analysis after stimulation with histamine was as follows:

	FREE HYDROCHLORIC ACID (°)
Fasting	0
First	0
Second	60
Third	76
Fourth	44

Wassermann and Kahn tests were negative. Hanger cephalin flocculation test was 3 plus. No urobilinogen was found in the urine.

Analysis of the dry solids (15.5 per cent) of a stool sample was as follows: total fat, 58 Gm. per 100 Gm.; free fatty acids, 43.5 Gm. per 100 Gm.; soaps, 1.9 Gm. per 100 Gm.; total split, 45.4 Gm. per 100 Gm.; neutral, 9.3 Gm. per 100 Gm.; unsaponified, 3.2 Gm. per 100 Gm.

Treatment.—This patient had never received liver extracts, yeast, or vitamin supplementation of his diet. He was placed on the preliminary sprue diet, high in carbohydrates and very low in animal proteins, and maintained on this diet throughout the experiment. On Sept. 16, 1946, when oral therapy with a concentrate of vitamin B₁₂ conjugate was initiated, the weight was 73 pounds and the hematologic findings were as follows: erythrocytes, 1,320,000 per cubic millimeter; hemoglobin, 7.2 Gm. per 100 c.c.; leucocytes, 1,400 per cubic millimeter; mean cell volume, 159 μ . No reticulocytes were observed. The sternal marrow showed 17.8 per cent megaloblasts.

The concentrate of vitamin B₁₂ conjugate used in therapy was supplied by Dr. J. J. Piffner and had a labeled potency of 1.2 mg. of potential folic acid per cubic centimeter. Of the total solids, approximately 10 per cent was vitamin B₁₂ conjugate, and less than 0.01 per cent was free folic acid. The concentrate was given orally in daily doses of 7 c.c., containing conjugated folic acid in an amount equivalent to about 8.4 mg. of free folic acid (23.2 mg. as pteroylhexaglutamylglutamic acid).

TABLE II. HEMATOLOGIC DATA OBTAINED IN PATIENT WITH SPRUE*

DATE	HEMOGLOBIN (GRAMS PER 100 C.C.)	ERYTHROCYTES (MILLIONS PER C.M.M.)	LEUCOCYTES (THOUSANDS PER C.M.M.)	RETICULO- CYTES (PER CENT)
9/16/46	7.2	1.32	1.4	0.0
9/17/46	8.7	1.06	2.1	0.0
9/18/46	6.0	1.21	2.4	0.0
9/19/46	5.2	1.13	1.0	0.0
9/20/46	4.8	1.07	0.9	1.4
9/21/46	--	--	--	3.4
9/22/46	--	--	--	14.0
9/23/46	5.8	1.27	3.0	23.8
9/24/46	5.8	1.31	1.7	28.4
9/25/46	5.0	1.56	1.8	33.0
9/26/46	6.0	1.75	2.4	30.4
9/27/46	6.4	1.86	3.3	24.0
9/28/46	7.2	2.14	4.7	23.8
9/30/46	6.8	1.95	2.2	23.6
10/ 1/46	6.4	2.15	3.1	20.0
10/ 2/46	6.6	2.02	3.5	15.2
10/ 8/46	6.4	1.89	6.0	9.4
10/14/46	7.8	2.05	6.0	7.4

*The patient (J. Q.) was given daily oral doses of a concentrate of vitamin B₁₂ conjugate (equivalent to 8.4 mg. of folic acid), beginning September 16 and continuing through Sept. 30, 1946 (15 doses). Additional therapy with liver extract (1 c.c. daily, containing 15 units) was begun on September 26 and was continued through Oct. 3, 1946. Folic acid was begun on October 3 at a level of 10 mg. daily for one week and 2.5 mg. daily thereafter.

Reticuloeytes began to appear in the peripheral blood on the fourth day of treatment (1.4 per cent); the following levels were encountered subsequently: 3.4 per cent (fifth day), 14 per cent (sixth day), 23.8 per cent (seventh day), 28.4 per cent (eighth day). A reticuloeyte peak of 33 per cent was encountered on the ninth day of treatment. On the tenth day daily subcutaneous injections of 1 c.c. of purified liver extract (15 units per cubic centimeter) were begun. At this time the weight was 73 pounds and the hematologic data were as follows: erythrocytes, 1,760,000 per cubic millimeter; hemoglobin, 6.0 Gm. per 100 c.c.; leucocytes, 2,400 per cubic millimeter; reticuloeytes, 30.4 per cent; and mean cell volume, 142 μ . The subjective and clinical improvement was even more evident in this patient than in the one who received the smaller doses of pteroylglutamate.

Four days after the institution of treatment, the patient felt better, his appetite had improved, the tongue showed newly formed papillae, and the stools had diminished in number and had improved in consistency. At this time the megaloblasts in the sternal marrow were only 0.6 per cent. On the eighth day of treatment the stools became formed, and since then there have been only one or two bowel movements daily.

URINARY FINDINGS

Studies of the urinary elimination of folic acid by normal human subjects, and by those with various pathologic conditions, have shown that the daily excretion of the vitamin rarely exceeds 5 μ g and usually amounts to from 2 to 4 μ g, when the intake is restricted to ordinary dietary sources of the vitamin (and to the intestinal synthesis that presumably occurs). Oral or parenteral administration of synthetic folic acid leads to a prompt augmentation

TABLE III. FOLIC ACID EXCRETION IN URINE OF PATIENTS WITH TROPICAL SPRUE

PATIENT M. Q. (FERMENTATION FOLIC ACID)				PATIENT J. Q. (CONJUGATED FOLIC ACID OF YEAST)			
DATE	URINE VOLUME (C.C.)	L. CASEI ASSAY (μ G./C.C.)	OUTPUT OF FOLIC ACID (MG./DAY)	DATE	URINE VOLUME (C.C.)	L. CASEI ASSAY (μ G./C.C.)	OUTPUT OF FOLIC ACID (MG./DAY)
6/19-20	1,760	--	<0.0002	9/15-16	990	0.0008	0.0008
6/20-21	1,180	--	<0.0002	9/16-17	--	--	--
6/21-22	2,210	--	<0.0002	9/17-18	850	0.0038	0.0032
6/22-23	890	--	<0.0002	9/18-19	880	0.0015	0.0013
6/23-24	1,560	--	<0.0002	9/19-20	630	0.013	0.008
6/24-25	1,330	0.31	0.41	9/20-21	380	0.180	0.068
6/25-26	650	0.85	0.55	9/21-22	910	0.051	0.046
6/26-27	1,060	0.42	0.44	9/22-23	1,140	0.031	0.035
6/27-28	1,620	1.06	1.71	9/23-24	1,280	0.042	0.054
6/28-29	1,150	0.80	0.92	9/24-25	1,020	0.147	0.150
6/29-30	2,440	1.50	3.66	9/25-26	1,690	0.189	0.32
6/30-7/1	2,920	0.44	1.28	9/26-27	1,280	0.30	0.38
7/ 1-2	2,820	0.60	1.69	9/27-28	1,850	0.50	0.93
7/ 2-3	2,420	0.61	1.47	9/28-29	1,570	0.95	1.50
7/ 3-4	3,270	0.41	1.34	9/29-30	1,720	0.66	1.14
7/ 4-5	1,460	4.90	7.15	9/30-10/1	1,510	0.48	0.73
7/ 5-6	2,460	2.96	7.28				
7/ 6-7	2,140	0.84	1.79				
7/ 7-8	2,000	0.41	0.82				
7/ 8-9	2,520	0.0008	0.002				

Patient M. Q. was given intramuscular injections of fermentation folic acid, equivalent to 3.1 mg. of free folic acid, beginning June 24 and continuing through July 7, 1946 (14 injections). In addition, injections of liver extract (1 c.c. daily, containing 15 units) were begun on July 4.

Patient J. Q. was given daily oral doses of the conjugated folic acid of yeast, equivalent to 8.4 mg. of free folic acid, beginning September 16 and continuing through Sept. 30, 1946 (15 doses). In addition, injections of liver extract (1 c.c. daily, containing 15 units) were begun on September 26.

Additional assays with *Str. faecalis* were carried out on all samples of urine obtained from one patient (M. Q.) and on several samples of urine from the other patient (J. Q.). The assay values found were in all cases in close agreement with those obtained with *L. casei*, a circumstance which indicates that the microbiologically active material in the urine of both patients was free pteroylglutamic acid.

of the amount of the vitamin in the urine and, depending on the size of the dose, to an elimination within twenty-four hours of from 15 to 75 per cent of the administered material. A normal subject given vitamin B₉ conjugate intramuscularly, in an amount equivalent to 1 mg. of free folic acid, on each of two successive days, excreted 8.3 per cent in the urine as the free vitamin; the same individual excreted 16 per cent when daily doses of 1 mg. of synthetic folic acid were given intramuscularly. On a dosage regime of about 10 mg. daily, either orally or parenterally, the excretion of the free vitamin in the urine usually ranges between 35 and 50 per cent.

In Table III data are presented on the urinary excretion of free folic acid by the patients who were given fermentation folic acid and the conjugated folic acid of yeast, respectively. Although only those data obtained with *L. casei* are presented in the table, duplicate analyses were run with *Str. faecalis* (*Str. lactis* R) on all urine samples obtained from the patient given fermentation folic acid and on occasional urine samples from the patient given the heptaglutamate. The very close agreement obtained through the use of the two organisms made it clear that the microbiologically active material present in the urine of both patients was pteroylglutamic acid. Treatment of the urine with preparations of conjugase gave no evidence of the presence in the urine of any conjugated forms of folic acid. The conjugases used exerted their expected activity when their effect on added conjugate was tested in the presence of urine from the patients studied.

DISCUSSION

It is of interest to comment on the remarkably small amount of folic acid excreted, prior to therapy, in the urine of the patients on the preliminary sprue diet. In one of these patients (M. Q.) the amount of folic acid excreted was below that which could be determined by the microbiologic method employed; in the other patient (J. Q.) the amount of vitamin excreted (0.8 μ g.) on one day was considerably smaller than is usually encountered in the urine of human subjects. A level of excretion of folic acid, below that easily determined, has also been observed in another patient with sprue on the same diet. It is not claimed that these unusually small amounts of urinary folic acid are necessarily characteristic of sprue since they may reflect the very small amount of the vitamin in the diet, but this diet is characteristic of that employed by those among whom sprue is found. Additional studies of the intake and excretion of folic acid of individuals in areas where sprue is encountered are greatly needed.

The data presented in Table III clearly indicate that pteroyldiglutamylglutamic acid served as a source of free folic acid in the patient with sprue (M. Q.) treated with this conjugate. However, the urinary excretion of the free vitamin was appreciably less during the first three days of therapy than on any of the subsequent days when the triglutamate was administered, a circumstance which suggests that retention was more marked when the need was greatest.

Particularly striking, however, is the relatively enormous excretion of folic acid that occurred during the first days of therapy with liver extract.

It should be pointed out that the daily dose (1 c.c.) of liver extract employed contained less than 0.001 mg. of microbiologically determinable folic acid, even after treatment with enzymes known to release the vitamin from its polyglutamates. Also, several investigators have shown that purified liver extracts comparable to this material exert no favorable effect in chicks, rats, or monkeys made deficient in folic acid. In view of the results previously mentioned, indicating that many patients with pernicious anemia are unable to utilize the conjugated folic acid of yeast, it seems possible that a factor of the liver extracts, presumably the factor concerned with the maturation of erythrocytes, markedly affected the metabolism of fermentation folic acid or of tissue complexes formed as a result of its administration. Since the patient was given daily doses equivalent to only 3.1 mg. of free folic acid, an excretion of over 7 mg. on each of two successive days suggests that stores of conjugated material were broken down to free folic acid much more rapidly than was the case prior to therapy with liver extract, although other explanations must be considered. This patient previously had received intermittent injections of liver extract, the last dose having been given only fourteen days prior to the first dose of fermentation folic acid; further, the presence of hydrochloric acid in the stomach is proof that achylia gastrica did not exist. The presence of a macrocytic anemia that responded to therapy demonstrates that during the pre-treatment period the patient failed either to absorb or to synthesize adequate amounts of the erythrocyte maturation factor. These data strongly suggest that the metabolism of this conjugate of folic acid in sprue is altered significantly by the administration of large doses of a liver extract high in antipernicious anemia activity and itself effective in the treatment of sprue. Since this patient had recently been given injections of liver extracts, it will be necessary to study a hitherto untreated patient in order to demonstrate beyond question that fermentation folic acid can be utilized in sprue without the participation of exogenous factors of liver extract. It will be necessary also to show that therapy with liver extract, after a preliminary period on synthetic folic acid alone, does not augment the urinary excretion of folic acid. Preliminary data obtained in patients with pernicious anemia do not indicate that liver extracts have such an effect.

It is not easy to interpret the findings in the patient with sprue (J. Q.) given pteroylhexaglutamylglutamic acid. However, one observation of considerable significance may be made, namely, that a striking clinical response was obtained with this conjugate, without urinary evidence for the cleavage of other than small amounts of the polyglutamate into free folic acid. The degree of reticulocytosis observed at the peak was maximal for the initial erythrocyte count of 1,320,000 per cubic millimeter of blood. It will be noted that on the seventh day, when the reticulocytes had already risen to about 24 per cent, the urinary excretion of folic acid was only about 54 micrograms. Whether these findings indicate that the conjugate was being utilized as such, or that the folic acid released from the conjugate was unusually well retained, cannot now be decided. That the components of the concentrate were poorly absorbed from the gastrointestinal tract is possible; however, the prompt and strik-

ing improvement in the clinical and hematologic condition of the patient, resulting from oral therapy with the conjugate, indicates that absorption was probably quite complete.

Following the parenteral administration of liver extract, in addition to oral dosage with the concentrate of heptaglutamate, urinary excretion of folic acid increased markedly, although not as promptly or as strikingly as in the patient given fermentation folic acid. In fact, from the data available, it is not possible safely to attribute the marked increase in folic acid excretion during the last four days of the experiment, entirely to the effect of the liver extract on the metabolism of the conjugate. The progressive increase in the daily excretion of free folic acid, which was observed prior to the administration of liver extract, suggests the possibility that the later effect might have occurred in the absence of the liver factor. In the light of other observations, however, it appears more likely that a factor of highly purified liver extract, presumably the erythrocyte maturation factor, influenced the ability of this patient with sprue to release folic acid from the conjugate of yeast or from complexes stored in the tissues. If such an assumption is justified, it follows that one of the biochemical defects resulting from a deficiency of the erythrocyte maturation factor may be an abnormality in the derivation of folic acid from its conjugates; it is possible that this abnormality may lie in the mechanism through which the body copes with inhibitors of the enzymes responsible for the release of the vitamin from its naturally occurring complexes. A defect of this type could be either partial or complete, possibly reflecting the fact that variable degrees of deficiency of intrinsic factor and probably of erythrocyte maturation factor are encountered.

SUMMARY

Following the parenteral administration of fermentation folic acid (pteroyldiglutamylglutamic acid) in a dose equivalent to 3.1 mg. of pteroylglutamic (folic) acid daily, the clinical and hematologic manifestations of sprue were rapidly and strikingly improved in a patient previously treated with liver extracts. Urinary studies showed that the patient excreted, prior to treatment, amounts of the free vitamin significantly lower than those usually encountered in normal subjects or those with pernicious anemia. Administration of the conjugate caused the elimination in the urine of free folic acid in an amount indicating that a very large proportion of the conjugate was converted *in vivo* to the free vitamin, although the amounts excreted during the first three days of therapy suggested that greater retention occurred when the need was greatest. When purified liver extract free of folic acid was administered, in addition to fermentation folic acid, a remarkable increase in the urinary excretion of free folic acid occurred, a level of approximately 7 mg. daily was maintained for two days, a circumstance which suggests that the erythrocyte maturation factor may be concerned with the release of stored complexes of folic acid or that in some other manner it affects the metabolism of folic acid.

Oral administration of a concentrate of vitamin B₆ conjugate (pteroyl-hexaglutamylglutamic acid) of yeast, in daily doses equivalent to 8.4 mg. of free pteroylglutamic (folic) acid, caused rapid clinical improvement in a patient with sprue given no previous treatment with liver extracts, yeast, or vitamins. The reticulocyte response was maximal for a patient with an initial erythrocyte count of 1,320,000 per cubic millimeter. Urinary studies showed at first only small increases in the excretion of the free vitamin, although the amount gradually increased to 0.32 mg. on the ninth day following initiation of therapy. Whether the small amounts excreted are indicative of utilization of the conjugate as such, or whether they suggest an unusually marked retention of free folic acid released from the conjugate, cannot be determined at present. Poor absorption is unlikely in view of the prompt and striking clinical and hematologic improvement observed. Following the administration of purified liver extract (free of folic acid), in addition to the conjugated vitamin, the daily urinary elimination of folic acid was increased to 0.93, 1.50, and 1.14 mg., respectively, on the second, third, and fourth days of additional therapy with the liver extract. Although these findings do not offer conclusive evidence, they strongly indicate that augmentation of the urinary elimination of free folic acid resulted from an effect of a component of purified extracts of liver on the metabolism of this naturally occurring complex of folic acid or on tissue complexes stored as a result of therapy with the conjugate.

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SULFACARBOXYTHIAZOLE: ABSORPTION, EXCRETION, TOXICITY, AND THERAPEUTIC RESULTS IN BACILLARY DYSENTERY AND NONSPECIFIC DIARRHEA

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THE sulfonamides have been classified as absorbable or poorly absorbable. Depending upon whether or not they pass through the gastrointestinal wall following oral administration. Poorly absorbable sulfonamides may be superior to those which are more readily absorbed in the treatment of enteric infections caused by sulfonamide-sensitive bacteria and for the preparation of patients who are to undergo bowel surgery for two reasons: first, the poorly absorbable sulfonamides are present in high concentrations within the intestines and, second, most of the systemic toxic reactions are avoided when the poorly absorbable compounds are used.

Sulfacarboxythiazole (2-sulfanilamido-5-carboxysulfathiazole)* is a recently synthesized compound in which a carboxy group is substituted on the thiazole ring of sulfathiazole. It differs from previously synthesized poorly absorbable sulfonamides such as succinylsulfathiazole and phthalysulfathiazole in that these latter drugs are made by the substitution of either a succinyl or phthalyl radical on the para-amino group. The activity of these drugs is dependent on their breakdown in the bowel to sulfathiazole, this being accomplished in the case of sulfacarboxythiazole by decarboxylation and in the case of the other two compounds by hydrolysis. When determinations for sulfacarboxythiazole are made on blood, feces, and urine, the designation "free drug" denotes the amount of sulfathiazole and sulfacarboxythiazole present. The designation "total drug" indicates the quantity of free drug plus the amount of the conjugated or bound form. The latter form of the drug consists of acetylsulfathiazole and acetylsulfacarboxythiazole.

Poth and Ross¹ have shown that phthalysulfathiazole in one-half the dosage was as effective as succinylsulfathiazole in the alteration of the coliform flora of the bowel of man. In equivalent doses the two drugs gave similar low blood concentrations (1 to 1.5 mg. per 100 c.c.) with 10 per cent of the succinylsulfathiazole recovered in the urine, while only 5 per cent of the phthalysulfathiazole was similarly recovered. White and associates² found sulfacarboxythiazole to be similar to phthalysulfathiazole with respect to solubility, absorption, and in vitro bacteriostatic action against dysentery bacilli. Other studies³⁻⁴ have shown sulfacarboxythiazole to be poorly absorbable, equally as bacterio-

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static as sulfaguanidine in vitro and in vivo, as well as nontoxic in man and animals. These preliminary data warranted a clinical study of this drug, the results of which we are reporting in this paper.

ABSORPTION AND EXCRETION

Sixty subjects were given sulfacarboxythiazole in doses varying from 0.1 to 0.5 Gm. per kilogram of body weight per day for periods of three to fifteen days. The total daily dose was calculated for each subject. One-half of this amount was given initially, followed by one-sixth at four-hour intervals. The free and total blood sulfonamide concentrations were determined daily by the method of Bratton and Marshall.⁵ Blood was taken for this purpose twenty-five to twenty-seven hours after the initial dose of sulfacarboxythiazole had been administered and at twenty-four hour intervals thereafter. Blood was obtained from two of the subjects four hours after sulfacarboxythiazole had been administered, and the drug was found to be present at that time.

Since the free and total concentrations were essentially the same in over 90 per cent of the determinations, only the total values are recorded. Among the few instances in which the conjugated form was present in significant amounts, it was never found to comprise more than 19 per cent of the total amount of the drug present. In Table I are listed the range of the total blood concentrations found for each dosage schedule. Ninety per cent of the blood concentrations were less than 1 mg. per 100 c.c., although concentrations as high as 2.7 and 4.3 mg. per 100 c.c. were observed in isolated instances. There was no relationship between the duration of treatment and the height of the blood concentrations. In several subjects the highest concentrations were found after two or three days of treatment, whereas lower concentrations occurred later in the course of the study. In general, higher blood concentrations were found in the group of subjects who received the larger doses. Traces of the drug were found in the blood for one to three days after the drug had been discontinued.

Four subjects, two of whom had bacillary dysentery and two of whom were healthy individuals, were given sulfacarboxythiazole for 40 to 112 hours. The total daily dose was calculated on the basis of 0.25 Gm. per kilogram of body weight. This was divided into six equal doses which were given at four-hour intervals. The initial dose was one-half the total daily dose. No restrictions were placed on the intake of food and fluid on any of the subjects, except that the patients with dysentery were maintained on a liquid diet for the first twenty-four hours. Before the drug was administered each subject evacuated

TABLE I. BLOOD CONCENTRATIONS FOLLOWING ADMINISTRATION OF VARIOUS DOSAGES OF SULFACARBOXYTHIAZOLE

DOSAGES (GM./KG. OF BODY WEIGHT)	BLOOD LEVELS (MG./100 C.C.)	NUMBER OF DETERMINATIONS
0.10	0.00 to 1.2	88
0.20	0.45 to 1.0	7
0.25	0.16 to 4.3	8
0.50	0.27 to 2.7	2

TABLE II. AMOUNTS OF SULFACARBOXYTHIAZOLE EXCRETED IN FECES AND URINES OF HUMAN SUBJECTS FOLLOWING ORAL ADMINISTRATION

Part I						
SUBJECT	DAY OF STUDY	GRAMS ADMINISTERED	GRAMS RECOVERED			
			FECES		URINE	
			FREE	TOTAL	FREE	TOTAL
H. D.	1	18	15.0	15.0	0.65	0.72
	2	9	9.0	9.0	0.36	0.46
	3		0.72	0.72	0.10	0.13
	4				0.16	0.26
	5				0.02	0.28
B. O.	1	15	9.4	10.0	0.50	0.60
	2	15	14.4	15.0	0.65	0.81
	3	15	10.9	10.9	0.60	0.69
	4	5	5.5	5.7	0.30	0.39
	5		0.078	0.078	0.045	0.57
W. D.	1	12	0.89	0.89	0.21	0.227
	2	18	13.5	13.9	0.35	0.44
	3	18	11.2	11.3	0.35	0.40
	4	18	16.7	16.8	0.34	0.40
	5	12	12.1	12.1	0.28	0.35
	6		12.6	12.61	0.16	0.21
	7		0.013	0.018	0.02	0.035
	8				0.013	0.015
G. L.	1	22	No stool	No stool	0.64	0.67
	2	24	22.0	22.25	1.1	1.4
	3		20.0	20.0	0.4	0.7
	4		0.1	0.1	0.3	0.4
	5				0.1	0.1
Part II						
SUBJECT			H. D.	B. O.	W. D.	G. L.
A. Recovered from feces						
Grams free			24.72	40.278	66.563	42.10
Grams total			24.72	41.678	67.168	42.35
Per cent of administered drug			91.55	83.35	86.11	92.06
Per cent conjugated			0	3.4	0.9	0.5
B. Recovered from urine						
Grams free			1.290	2.095	1.733	2.54
Grams total			1.599	2.547	2.077	3.27
Per cent of administered drug			5.92	5.10	2.66	7.11
Per cent conjugated			19.5	17.7	16.5	22.3
C. Recovered from feces and urine						
Grams free			26.010	42.373	69.296	44.64
Grams total			26.319	44.225	69.245	45.62
Per cent of administered drug			97.47	89.45	99.17	99.17
Per cent conjugated			0.78	4.4	1.3	2.1

his bowels and bladder. The total daily stools and urines were collected thereafter and analyzed for free and total sulfonamide content until the drug was no longer detectable in the excreta.

The technique of these determinations was as follows: The total daily feces were emulsified in 1,000 to 2,000 c.c. of tap water and filtered several times through cotton gauze. An aliquot portion was diluted anywhere from 1:3 to 1:500 with tap water depending on the concentration of sulfonamide anticipated. This solution was tested for its sulfonamide content by the method

of Bratton and Marshall.⁵ Appropriate dilutions of samples of the total daily urine were similarly analyzed for their sulfonamide content.

The results of the excretion studies on each subject are shown in Table II. Although most of the drug was excreted during the course of administration, detectable concentrations were found in the excreta for two or three days after sulfacarboxythiazole had been discontinued. The percentages of the drug recovered in the stools were between 83.4 and 92.1 per cent, of which 3.4 per cent or less was in the conjugated form. In the urines the amounts recovered varied from 2.7 to 7.1 per cent, with 16.5 to 22.3 per cent occurring as the conjugated form. The total amounts recovered from both excreta ranged from 88.5 to 99.2 per cent of the amounts administered. The blood concentrations are reported in Table I.

BACTERIOLOGIC STUDIES

As part of the investigation of sulfacarboxythiazole, a study of its antibacterial activity on the intestinal flora of twenty subjects was made. The number of colonies of coliform organisms was used as an index of the intestinal population. Either the total amount of feces passed in twenty-four hours or a single specimen was weighed and then suspended in 1,000 to 2,000 c.c. of sterile isotonic salt solution. A series of tenfold dilutions were then made of this suspension. These diluted specimens were mixed in pour plates with tryptose-phosphate agar containing 5 mg. per 100 c.c. of para-aminobenzoic acid. The plates were incubated overnight at 37° F., after which time the colonies were counted and the number calculated for each gram of feces. The number of coliform colonies per gram present on the day treatment with sulfacarboxythiazole was started and thereafter, including the first few days after treatment had been discontinued, are shown in Table III. In each instance a control culture taken the day before treatment revealed innumerable colonies of coliform organisms. Within twenty-four hours there was considerable decrease in the number of colonies. Thereafter the cultures were sterile or only occasional colonies were observed, until the drug was discontinued, after which the coliform population steadily increased.

CLINICAL STUDY

Fifty patients with diarrhea were treated with sulfacarboxythiazole. Shigellae were isolated in thirty patients and salmonellae in two, and in the case of eighteen patients the causative organism was undetermined. Forty patients were below the age of 12 years; the other ten were between 22 and 54 years of age. There was no evidence of any other disease in any of the patients in this series. The total daily dose employed for the children was calculated on the basis of 0.1 Gm. per kilogram of body weight. The initial dose was equivalent to one-half the total daily dose, and thereafter the calculated dose was divided into six doses administered every four hours. The only other therapeutic measures employed were restriction of diet and administration of fluids parenterally when necessary.

TABLE III. EFFECT OF SULFACARBOXYTHIAZOLE ON INTESTINAL BACTERIAL POPULATION USING COLIFORM ORGANISMS AS AN INDEX
(NUMBER OF COLONIES OF COLIFORM ORGANISMS PER GRAM OF FECES)

PATIENT	DAYS AFTER SULFACARBOXYTHIAZOLE STARTED																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
W.M.	21	22	0	2,100	1														
G.S.	1	0	0	5,000	120,000	I													
G.R.	2	1	0	1,200	2,000	1,800,000	I												
E.R.	4	0	0	1,120	440,000	6,800,000	I												
H.D.	314	28	0	000	780,000	2,700,000	I												
R.W.	1	0	2	12,000	680,000	4,000,000	I												
J.B.	13	4	0	560	160,000	900,000	4,000,000	I											
L.P.	5	2	2	4	60	I													
G.L.	100	0	0	0	670	140,000	900,000	I											
C.N.	280	59	50	0	2,400	800,000	2,000,000	I											
G.B.	10	13	9	0	2,900	658,000	4,840,000	I											
J.H.	26	3	0	31	3,260	634,000	1,520,000	I											
A.R.	1	0	0	0	0	15,800	995,000	I											
W.K.	51	0	0	0	0	2	960	1,600,000	5,200,000	I									
L.W.	6	0	0	0	0	0	420	1,200,000	I										
W.D.	3	0	0	21	0	7	108	17,000	1,900,000	I									
G.D.	60	41	21	2	0	0	960	1,600,000	4,740,000	I									
L.P.	2	0	0	0	0	0	0	9,500	380,000	650,000	1,980,000	I							
F.H.	107	63	0	0	0	0	0	1,000	66,000	3,100,000	I								
B.S.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2,000	160,000	I

I, Immurable colonies.

Heavy line indicates time at which sulfacarbonythiazole was discontinued.

TABLE IV. RESULTS OF SULFACARBOXYTHIAZOLE THERAPY IN FIFTY PATIENTS WITH DIARRHEA

CAUSATIVE ORGANISM	NUM- BER OF PA- TIENTS	DAY OF TREAT- MENT ON WHICH RECTAL CULTURES BE- CAME FREE OF PATHOGENS				DAY OF TREATMENT ON WHICH DIARRHEA ABATED					NO DIAR- RHEA	DAY OF TREATMENT ON WHICH DIARRHEA ABATED					NO RE- SPONSE	DAY OF TREAT- MENT ON WHICH PATIENTS BECAME AFEBRILE			
		1	2	3	4	1	2	3	4	5		1	2	3	4	5		AFE- BRILE	1	2	3
<i>Shigella dysenteriae</i>																					
Flexner	17	13	1		3		9	3	2	1		2					2	3	11	2	1
Newcastle	8	4	1	3		1	6	1										3	5		
Sonne	5	3	1	1		1	2										2	1	4		
Salmonellae	2	1			1	2												1	1		
Unidentified	18						11	3	1			1					2	4	12	1	1

The results of the administration of sulfacarboxythiazole in these fifty patients are shown in Table IV. In the group with specific infections, the causative organism could no longer be cultured from the stools after the fourth day of treatment and throughout the observation period of one to two weeks after treatment had been discontinued. Of the forty-six patients who had diarrhea, forty were free of this symptom by the fifth treatment day. For the other six patients the treatment was considered a failure because the diarrhea persisted in spite of the fact that sulfacarboxythiazole was administered for eight days. It is of interest, however, that the stools of four of the patients in the latter group contained pathogenic organisms before therapy was started and these organisms had disappeared from the stools by the fourth day of treatment. All of the thirty-eight patients whose temperatures were 99° F. or higher at the time treatment was begun were afebrile by the fourth day.

TOXICITY

Among the sixty persons who received the drug, six developed toxic reactions. Four patients developed diarrhea. Three of these were receiving amounts totaling 0.5 Gm. per kilogram of body weight per day and the other patient 0.25 Gm. per kilogram of body weight per day. The diarrhea stopped when the doses were decreased. One patient developed nausea and vomiting following the ingestion of the initial dose consisting of 14 tablets (7 Gm.) or 0.25 Gm. per kilogram of body weight. The sixth patient developed a nodular rash similar to those which occur following the administration of sulfathiazole. The rash disappeared promptly after the drug was discontinued.

No abnormal findings were observed in the blood or urine of any of the subjects, although these were examined at frequent intervals during the course of study.

DISCUSSION

Sulfacarboxythiazole is a poorly absorbed rather than a completely non-absorbable sulfonamide compound. In a series of 105 determinations, only occasionally was the total of the free and acetylated drug above 1 mg. per 100 cubic centimeters. Sulfacarboxythiazole was recovered from the urine of several subjects in amounts up to 7.1 per cent of the total amounts administered.

Additional evidence that sulfacarboxythiazole was occasionally absorbed in significant amounts was the nodular rash which was observed in the case of one patient. From these studies it appears, however, that almost all the drug is available in the active form in the bowel, since less than 3.4 per cent of the amounts recovered in the feces were in the conjugated form.

The results of this study of the absorption and excretion of sulfacarboxythiazole are consistent with those reported by Harris and Finland.³ These investigators found that blood concentrations were usually below 1 mg. per 100 c.c. but that concentrations up to 2 mg. per 100 c.c. were occasionally observed. Whereas amounts of 83.4 to 92.1 per cent of the drug were recovered in the feces of our patients, Harris and Finland³ found only 54.6 to 82.9 per cent. Furthermore, the amounts which they demonstrated in the urines of their group of patients were higher, 9 to 23 per cent, in comparison to recovery amounts of up to 7.1 per cent in our series. In both series only small amounts of the conjugated form of the drug were present in the feces.

TABLE V. RESULTS OF SULFADIAZINE THERAPY IN FIFTY PATIENTS WITH DIARRHEA

CAUSATIVE ORGANISM	NUMBER OF PATIENTS	DAY OF TREATMENT ON WHICH RECTAL CULTURES BECAME FREE OF PATHOGENS				DAY OF TREATMENT ON WHICH DIARRHEA ABATED					DAY OF TREATMENT ON WHICH PATIENTS BECAME AFEBRILE			
		1	2	3	4	1	2	3	4	NO RESPONSE	AFEBRILE	1	2	3
<i>Shigella dysenteriae</i>														
Flexner	24	15	6	2	1	10	8	1	3	2	4	12	6	2
Newcastle	8	6	1	1		7		1			6	1	1	
Sonne	3	2	1			1	1	1			1	2		
Unidentified	15					4	4	3	2	2	5	6	2	2

Sulfacarboxythiazole has been found to be satisfactory in the treatment of fifty patients with diarrhea. The results of treatment in this group were compared with the results (Table V) observed in fifty patients with similar illnesses who were treated with sulfadiazine prior to the period that sulfacarboxythiazole was employed. Stool cultures became negative within four days in both groups. Ninety-two per cent of the sulfadiazine-treated patients were free of diarrhea by the fourth treatment day, while 87 per cent were similarly improved when treated with sulfacarboxythiazole. This difference is not considered significant. Four sulfadiazine-treated and six sulfacarboxythiazole-treated patients continued to have diarrhea with negative stool cultures even though the drug was continued for eight days. Of the failures in the latter group, four improved on a course of sulfadiazine, although two of these patients had not responded at first to this drug. Sulfadiazine was ineffective in the two remaining patients who had failed to improve when they were given sulfacarboxythiazole. All patients in both groups who had elevated temperatures at the time treatment was started were afebrile within three days.

The only other clinical evaluation of this sulfonamide was reported by Harris and Finland.³ They noted prompt recovery in five patients with dysentery following the administration of sulfacarboxythiazole.

In anticipation of the use of sulfacarboxythiazole in the preparation of patients for bowel surgery, stool cultures on plain agar plates were taken daily on twenty subjects. The prompt disappearance and continued absence of most of the intestinal flora from the stools while the drug was continued would make it appear that the drug is effective as an intestinal antiseptic.

SUMMARY AND CONCLUSIONS

1. Sulfacarboxythiazole (2-sulfanilamido-5-carboxythiazole) is a poorly absorbed sulfonamide when given orally. Blood concentrations were usually below 1 mg. per 100 cubic centimeters. The excretion of the drug in the stools varied between 83.4 and 92.1 per cent of the amount ingested, while the urinary excretion ranged from 2.7 to 7.1 per cent of the oral dose.

2. Sulfacarboxythiazole gave satisfactory results in forty-four out of fifty patients with diarrhea. The results of this treatment compare favorably with the results of sulfadiazine therapy in a similar group of fifty patients.

3. Six patients developed toxic reactions from sulfacarboxythiazole. Gastrointestinal symptoms developed in five patients, in four of whom the large doses administered were undoubtedly responsible. A sixth patient developed a nodular skin rash.

4. Sulfacarboxythiazole appears to be a useful sulfonamide in the treatment of enteric infections caused by sulfonamide-sensitive bacteria and deserves a trial in the preparation of patients who are to undergo bowel surgery.

We wish to thank Miss C. Barbara O'Neil for technical assistance.

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THE CONCENTRATION OF STREPTOMYCIN IN DOG BILE

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STREPTOMYCIN has been found to be effective against the intestinal types of microorganisms, including *Escherichia coli*, and the dysentery and typhoid organisms.

Reimann, Elias, and Price¹ and the Committee on Chemotherapeutic and Other Agents of the National Research Council² have used streptomycin in the treatment of patients with typhoid fever. No evidence was obtained proving that the duration or mortality rate was influenced because too few cases were studied.

Rutstein and associates³ studied the effect of streptomycin on three typhoid carriers. They found that 150,000 units given intramuscularly every three hours produced effective blood levels but did not affect the typhoid bacilli in the stool. Following the oral administration of 31,000 units every three hours, a definite transitory bacteriostatic effect on the typhoid bacilli in the stool was obtained. Typhoid bacilli grow well in bile, and organisms remaining in the gall bladder and in the bile may be responsible for the carrier state. However, Rutstein and his associates did not simultaneously use the oral and parenteral routes of administration.

The results of studies on the excretion of streptomycin in the bile of the rabbit and the human patient have been reported. Stebbins, Graessle, and Robinson⁴ found that rabbits excreted from 5 to 10 per cent of intravenously administered streptomycin. Zintel and co-workers⁵ gave 600,000 units intravenously to two human patients and obtained streptomycin levels in the bile of 8 units per cubic centimeter. Heilman and associates⁶ gave one patient 100,000 units intramuscularly every three hours for five days. They obtained bile concentrations as high as 13 units per cubic centimeter. The serum level at the same time was 6 units per cubic centimeter. It was concluded that the liver could concentrate the drug. Zaslow,⁷ in a preliminary report, administered streptomycin to patients before cholecystectomy and obtained considerable amounts of the antibiotic in gall bladder bile, if the cystic duct was patent. In the case of an obstructed cystic duct, no streptomycin was found in the gall bladder. Even though the drug remained in the gall bladder for a considerable length of time, he found no evidence of absorption or concentration. In intrahepatic and in obstructive jaundice, no antibiotic agent was excreted in the bile. In patients with normal livers, considerable amounts of streptomycin appeared in the hepatic bile; however, the level of streptomycin activity in the bile was less than that in the blood, indicating that the liver did not concentrate this anti-

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biotic agent. Zaslow did not, however, administer sufficient streptomycin to produce a high level in the serum and bile.

In this investigation we attempted to answer the following questions: (1) Can high levels of streptomycin be obtained in hepatic bile? (2) Can high levels of streptomycin be obtained in gall bladder bile? (3) Is streptomycin concentrated by the normal gall bladder?

EXPERIMENTAL

Five dogs were anesthetized with pentobarbital. The common bile duct was cannulated, and the cystic duct was clamped off.

During the course of twenty minutes, streptomycin was given by intravenous drip in amounts ranging from 187,000 to 375,000 units. In Table I are given the results for a typical dog.

In an attempt to ascertain whether the liver would concentrate the drug, one dog was treated similarly, and simultaneous blood and hepatic bile levels were obtained. The results are shown in Fig. 1.

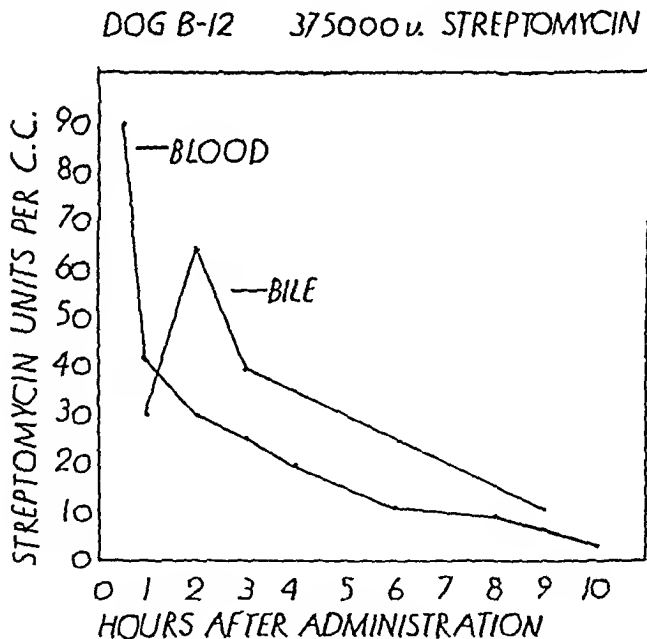


Fig. 1.

The concentration of streptomycin in gall bladder bile was investigated in six unanesthetized dogs which received the drug by intravenous drip and which were anesthetized one hour after the administration of the drug. Three of the dogs were in a fasting state, and three others received a meal of egg yolks and cream five hours before the experiment. The results are shown in Table II.

Twelve more dogs received streptomycin intramuscularly while in the fasting state. Three were given a single injection at 7 P.M. on the first day. At 11 A.M. on the second day, the animals were anesthetized and their gall bladder

content was removed. The other nine dogs received a total of seven injections each, four at three-hour intervals beginning at 10 A.M. on the first day and three more injections beginning at 10 A.M. on the second day. The animals were anesthetized at 11 A.M. on the third day. The results are shown in Table III.

TABLE I. DOG A-2 (187,500 UNITS OF STREPTOMYCIN)

MINUTES AFTER STARTING INTRAVENOUS DRIP	BILE VOLUME (C.C.)	STREPTOMYCIN CONCENTRATION IN BILE (UNITS PER C.C.)	TOTAL STREPTOMYCIN (UNITS)
Control	3.7	0	0
30	4.3	6	26
60	3.0	50	150
120	4.6	135	621
165	2.9	108	313

TABLE II.

DOG	STREPTOMYCIN (UNITS)	MEAL	STREPTOMYCIN CONCENTRATION IN BILE (UNITS PER C.C.)
B-1	50,000	None	1.2
B-2	50,000	None	0.6
B-3	50,000	Egg yolks and cream	0
B-6	50,000	Egg yolks and cream	0
B-10	375,000	None	22
B-11	375,000	Egg yolks and cream	25

TABLE III.

NUMBER OF DOGS	NUMBER OF INTRAMUSCULAR INJECTIONS	TOTAL AMOUNT OF STREPTOMYCIN RECEIVED (UNITS)	AVERAGE VOLUME OF GALL BLADDER BILE (C.C.)	AVERAGE STREPTOMYCIN CONCENTRATION (UNITS PER C.C.)
1	1	100,000	11	<2
2	1	160,000	20	2
3	7	140,000	11	<2
3	7	280,000	17	2.5
3	7	420,000	10	4.5

DISCUSSION

The results on the five dogs with a cannula in the common duet indicate that a maximum concentration of streptomycin in hepatic bile was reached in two or three hours. The highest level obtained, 135 units per cubic centimeter, was in Dog A-2 which received 187,500 units by intravenous drip in twenty minutes. Small amounts of streptomycin were still present in the bile after ten hours. The total amount excreted in the bile was only a fraction of a per cent of the amount administered.

The graph in Fig. 1 shows that two hours after starting the intravenous drip the serum concentration was 30 units of streptomycin per cubic centimeter while the concentration in hepatic bile was 64 units per cubic centimeter, indicating that the normal liver of the dog concentrates streptomycin.

The results in Table II indicate that 50,000 units given intravenously are inadequate to produce high levels in the gall bladder bile, irrespective of whether

a meal of egg yolks and cream was given or not. With 375,000 units, adequate levels of streptomycin in the gall bladder were obtained. Here also a meal of egg yolks and cream made little difference.

The results on the dogs which received streptomycin intramuscularly indicate that: (1) A single intramuscular injection of the drug will produce about as high a concentration in the gall bladder as will the same quantity divided into several doses; and that (2) the gall bladder does not appear to concentrate streptomycin since repeated intramuscular injections do not produce nearly as high a level in the gall bladder bile as does smaller doses given intravenously during the course of twenty minutes.

SUMMARY

1. Studies on streptomycin in hepatic and gall bladder bile of twenty-three dogs were conducted.

2. High levels of streptomycin were observed in both hepatic and gall bladder bile when relatively large doses were given slowly intravenously.

3. The liver was observed to concentrate streptomycin in one experiment.

4. The gall bladder does not concentrate streptomycin. The salt of streptomycin, being soluble in saline solution, is apparently absorbed along with water and electrolytes when the bile is concentrated by the gall bladder.

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CUTANEOUS TEST WITH COCCIDIOIDIN

REVIEW OF THE LITERATURE AND REPORT OF A SERIES IN TEXAS

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SINCE the original description of coccidioid granuloma in South America by Wernicke¹ and Posada² in 1892 and in North America by Rixford and Gilchrist^{3, 4} in 1894 and 1896, great advances have been made in our understanding of the nature of the disease,⁵ of its pathologic,^{6, 7} clinical,⁸⁻¹⁰ and epidemiologic¹¹ features, and especially of the diagnostic criteria involved. Among these criteria, one of the most objective and, therefore, reliable is the specific skin test with coccidioidin, which is regularly used in ruling coccidioidomycosis in or out in cases having a clinical picture compatible with the disease. Although the test is widely used, there has been to date no comprehensive review of the literature pertaining to it; it is the purpose of the present report to fill this need and to present the results of a series of coccidioidin tests performed in Dallas, Texas.

Cooke,^{12, 13} in 1914 and 1915, first undertook investigations sighted at the detection of agglutinins, precipitins, and complement-fixing substances in the serum of patients with coccidioid granuloma, and her work gave the impetus to other workers to investigate cutaneous reactions in this disease. Using antigens prepared from the mycelial growth on solid culture mediums and from the sporelike bodies present in the exudate from lesions, Cooke concluded that these antigens could not be used to demonstrate specific complement-fixing substances or agglutinins in the blood serum of patients nor did they produce any specific skin reactions. She found precipitins in serum diluted 1:160 and mixed with an extract of dried cultures. These studies were continued in 1916 by Cummins and Sanders¹⁴ who reported that, although they could not demonstrate agglutinins, precipitins, or complement-fixing substances in the serum of patients and animals with the disease, the concentrated filtrate from heated and unheated broth cultures caused a cutaneous reaction in some of their infected rabbits and to a lesser degree in normal rabbits. Davis¹⁵ obtained complement-fixing reactions with a concentrated culture as antigen and reported an intradermal skin reaction with killed homologous organisms definitely more marked than that obtained following the injection of a similar antigen made from cultures of *Sporothrix*, *Blastomyces*, or agar. Hirsch and Benson¹⁶ concluded from the known histologic reactions to *Coccidioides immitis*, with its similarity to the specific reaction to *Mycobacterium tuberculosis* and its lack of identity with a nonspecific foreign body reaction, that some soluble substance is produced by *C. immitis* which resembles,

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in some respects at least, the tuberculin produced by the tubercle bacillus and that skin reactions can be obtained in patients and animals infected with *C. immitis* which resemble those with tuberculin in tuberculous hosts. Using a modification of the medium used by Braun and Cahu-Bronner¹⁷ in their studies of general bacterial physiology, they¹⁸ found that growths of *C. immitis* in effect liberate a soluble specific substance causing skin reactions in infected animals and patients. Hirsch and Benson were the first workers to study the properties of this soluble specific substance. They found that it is not destroyed by heating to 80° C. for thirty minutes and that it is flocculated by electrodialysis. In 1928, da Fonseca and de Area Leão¹⁸ gave the first accurate description of a positive coccidioidin skin test. They pointed out that sensitive specific reactions are obtained to filtrates of *C. immitis* injected intradermally and that these reactions consist of an erythema accentuated at the inoculation site and a papular erythematous induration appearing several hours after injection and gradually increasing to a maximum in twelve to twenty-four hours. They further observed that the test may be accompanied by temperature elevation and focal reaction in patients with coccidioidal granuloma.

Beck, Traum, and Harrington¹⁹ further studied the problem in animals and raised the question of possible cross reactions with tuberculosis, reporting weakly positive tuberculin reactions in animals infected with *C. immitis*. Hurwitz, Young, and Eddie²⁰ also reported positive coccidioidin reactions in patients having no known coccidioidal infection. However, Gifford, Buss, and Douds,²¹ in an extensive survey in Kern County, California, and in studies of patients with San Joaquin fever, showed specificity of the coccidioidal reaction and were pioneers in reporting a high incidence of positive tests in individuals living in the San Joaquin valley. Dickson and Gifford,²² in 1938, following the preliminary work of Jacobson,²³ showed that cutaneous sensitivity to coccidioidin occurs six to seven days after the inoculation of guinea pigs and rabbits by chlamyospore inhalation. These authors²² stated that the active antigenic substance was not identified, questioning by implication the work of Hirsch and Benson¹⁶ (vide supra). They also made the observation that patients with valley fever react with a violent necrotizing response to the intradermal injection of 0.1 c.c. of filtrate, as advocated by Jacobson in a study the results of which were further corroborated by Dickson^{24, 25} in the same year, when he showed that the reaction of valley fever patients to coccidioidin was much more violent than that of patients with coccidioidal granuloma. This finding repudiated the preliminary impression of da Fonseca and de Area Leão¹⁸ (vide supra).

Kessel,²⁷ in 1939, showed that in the final stages of coccidioidal granuloma four weeks before death the skin test is negative but that other active cases of coccidioidal granuloma prior to the final stages give a positive skin reaction. He also demonstrated that patients with tuberculosis as a rule give negative reactions, that guinea pigs experimentally inoculated with *Myco. tuberculosis* give a negative coccidioidin skin test and a positive

tuberculin test, and that guinea pigs experimentally inoculated with *C. immitis* give a positive coccidioidin skin test and react negatively to tuberculin tests. By these experiments, Kessel furnished objective evidence to support the presumed high degree of specificity inherent in the coccidioidin skin test, a specificity which had already been suspected by Dickson.²⁸

The investigation of coccidioidin sensitivity in children and the correlation of positive tests with the existence of a syndrome consisting of fever, cough, aching, anorexia, sore throat, and pleural pain followed in from a few days to two and one-half weeks by erythema nodosum and positive sputum culture at the time of coccidioidin positivity, was undertaken in 1939 by Faber, Smith, and Dickson,²⁹ and their results checked closely with those anticipated on the basis of previous studies. In the following year, Smith³⁰ further studied the epidemiologic aspects of coccidioidomycosis by performing intradermal tests on healthy volunteers who had had clinical San Joaquin valley fever. These tests were all strongly positive; however, sensitivity to the antigen acquired from clinical infection could not be differentiated from sensitivity due to reinfection, since the subjects were residents of an endemic area. An isolated example suggesting strongly that sensitivity acquired during initial contact with the organism is permanent was cited. Smith's results indicated also that sensitivity to coccidioidin is established in from two to seventeen days following the onset of symptoms.

The geographic distribution of endemic centers of coccidioidal infection as determined by cutaneous test is listed by Davis, Smith, and Smith³¹ and according to these writers includes, besides the San Joaquin valley, Southern California, Arizona, Texas, and probably New Mexico and Mexico. Sporadic cases are also said to have been reported from Europe and from the Chaco region of South America. A new endemic area at Camp Roberts, California, was described in 1942 by Shelton,³¹ who, using the antigen supplied by Dr. C. E. Smith (the same antigen as was used in the series reported in the present paper), showed that of 736 soldiers who came from other parts of the United States to Camp Roberts fourteen became infected during three months' residence there. His original series of tests on 888 soldiers constituting a heterogeneous group chiefly from the Middle West (Illinois, Missouri, Nebraska, Arkansas, and Texas) resulted in eleven positive reactions or 1.2 per cent incidence of positivity. In the same year (1942), Aronson, Saylor, and Parr,³² working in the endemic area of Arizona, demonstrated presumptively that the occurrence of calcified pulmonary nodules may be due to pulmonary infection with *C. immitis*, using the coccidioidin skin test and the tuberculin test as criteria for evaluation of the roentgenograms. Previously, in 1939, Phillips³³ had drawn attention to possible endemicity of coccidioidal infection in Phoenix, Arizona, but he quoted a series too small to constitute more than suggestive evidence of such endemicity.

Smith³⁴ has interpreted the skin reaction to coccidioidin as a nonspecific allergic manifestation of a specific sensitization. He pointed out that the sensitivity to coccidioidin becomes established in clinical cases of primary

coccidioidomycosis just before the appearance of the characteristic erythema nodosum, which must be present in order to establish a diagnosis of valley fever. He points out, further, that this eruption may represent either an undulating allergy or an effect of a circulating antigen in a hypersensitive person.

Peers, Holman, and Smith³⁵ further demonstrated the usefulness of the cutaneous reaction to coccidioidin in differentiating coccidioidomycosis from pulmonary tuberculosis. In an epidemic of primary pulmonary coccidioidomycosis comprising seventy-five cases reported by Goldstein and Louie,³⁶ there was 100 per cent correlation between the clinical entity and the skin test. In a recent paper, Denenholz and Cheney³⁷ stress the diagnostic significance of the coccidioidin skin test, pointing out that a positive test probably has the same meaning in coccidioidomycosis as a positive reaction to tuberculin has in tuberculosis; it indicates that the patient has, or has had in the past, a coccidioidal infection, either clinical or subclinical, and does not necessarily imply that a positive reactor's present illness is due to infection with *C. immitis*. A negative coccidioidin skin test generally excludes the possibility of a coccidioidal infection, with only two possible exceptions, namely, the theoretic possibility that a patient with an active coccidioidal infection due to a strain not present in the antigen might react negatively to the test and the actual, but rare, possibility of negative reaction in a patient with severe disseminated coccidioidomycosis in a late anergic phase.

Emmons and Ashburn³⁸ have demonstrated that there is some cross sensitivity between *C. immitis* and a fungus isolated by them from rodents in Arizona which they have named *Haplosporangium parvum*. It may be that this cross sensitivity is responsible for the borderline positive reactions to coccidioidin commonly encountered in persons living in some areas in the Middle West, as pointed out by Smith.³⁴ In any case, the test may legitimately be considered highly specific in the overwhelming majority of cases, indicating coccidioidal infection, past or present.

REPORT OF A SERIES IN DALLAS, TEXAS

There is no report in the literature of any series of coccidioidin skin tests performed in Texas; yet from other series the impression has been gained that Texas possibly represents an endemic focus of coccidioidal infection. The present paper presents the results of an investigation undertaken in Dallas, Texas, with the purpose of determining the incidence of positive coccidioidin tests in an unselected series as far as probable or possible coccidioidin reactivity was concerned. In this study, 413 persons were tested. The subjects comprised 98 medical students, 102 student nurses, 202 patients on the wards of Parkland Hospital, and a miscellaneous group of 11 additional individuals. The following personal data were obtained from each subject before the test was performed: age, previous occupations, history of erythema nodosum, previous coccidioidin test with result, results, if known, of previous x-ray examination of the chest, home state, other places of residence, and places of travel.

The coccidioidin which was used in the performance of all the tests in the series was obtained from Dr. C. E. Smith of the Department of Public Health at Leland Stanford Junior University. The antigen, as supplied, was prepared by growing ten strains of fungus coccidioides in the Bureau of Animal Industry medium, the formula used for making tuberculin modified only in the use of 2.5 per cent glycerine rather than stronger concentrations. After the fungus had been grown for two months, it was Berkefeld filtered and merthiolate was added to a final concentration of 1:10,000. This material was subjected to sterility tests, animal inoculation, and tests on human beings of known sensitivity. The dilution used was 1:100 in sterile physiologic saline, and the tuberculin syringes used had never been used for any other procedure. Each needle used was employed for several consecutive tests without resterilization.

Each test consisted of the intradermal injection of approximately 0.1 c.c. of the diluted antigen into the skin on the volar aspect of the right forearm. Special care was taken that a wheal was raised at the site of injection in every member of the series, that is, that a true intradermal test was done. Each test was read in twenty-four and forty-eight hours. Any test showing a palpable induration at the injection site was considered to be a positive test; all others were recorded as negative. Grading of the tests was not attempted on the basis of standards any more minutely defined than those described.

On nine and eight, respectively, of the positive reactors to coccidioidin, simultaneous tuberculin and histoplasmin tests were done by McGolrick and Moore³⁹ as a part of an investigation undertaken to correlate skin tests with tuberculin and histoplasmin with thoracic roentgenogram evidence of pulmonary calcific nodules; the results are mentioned in the next section.

RESULTS OF THE TESTS

Of the 413 subjects, eleven (or 2.66 per cent) were positive reactors by the criteria which have been defined. This incidence of positivity is somewhat higher than that found by Shelton, whose group was, as noted, a homogeneous one taken chiefly from the Middle West, indicating that the incidence of sensitivity to coccidioidin is slightly greater than that encountered in Camp Roberts, California, prior to the establishment of endemicity there. No significant correlation between vocation and skin test positivity was obtained in the positive reactors; in fact, all but two of the reactors were persons whose occupations kept them indoors and hence were not especially liable to inhalation of chlamydospores of *C. immitis*.

Tuberculin tests were done on nine of the positive reactors as part of a series run simultaneously with the present one by McGolrick and Moore³⁹ on the nurse and medical student groups. To a 1:1000 dilution of tuberculin (O.T.), one subject reacted positively and one gave a doubtful reaction, all the remainder being negative; whereas in the case of a dilution of 1:100, there were one positive and three doubtful reactors. In histoplasmin tests done by the same workers on eight of the positive coccidioidin reactors, three were found to be positive. Only one of these simultaneously tested subjects showed a positive reaction to

both dilutions of tuberculin, to histoplasmin, and to coccidioidin, although another subject who gave a clearly positive reaction to coccidioidin and histoplasmin also reacted to tuberculin in both dilutions, though doubtfully according to the criteria adopted by McGilrick and Moore. In summary, the majority of of the coccidioidin-positive subjects in this series were found to be nonsensitive to antigens of tuberculosis and histoplasmosis, two common causes of pulmonary calcification.

Of the positive reactors, only two had been previously tested with the antigen, and their reactions had been positive, corroborating the work previously quoted that sensitivity to coccidioidin, once acquired, tends to persist.

The states of residence of the coccidioidin positive reactors were investigated, and in cases in which the person in question had been a resident at various times of several states, each of those states was recorded. It was found that Texas was at one time, past or present, the state of residence of 81.8 per cent of the individuals studied; California was the next most frequently encountered state of residence (45.4 per cent), and Arizona was the third in frequency with 36.3 per cent. Some significance can thus be attached to the fact that most of the positive coccidioidin reactors had at one time lived in one of several well-known endemic areas. The fact that the overwhelming majority had lived in Texas is, of course, a factor of the locale in which the survey was undertaken. Territories in which the members of the positive reactor group had traveled were also surveyed, and only periods of travel were included in which the individuals had remained in the various locales for at least two weeks. That three had traveled in Mexico is of interest in the light of the suggestive evidence that there exists lurking endemicity in that country,¹⁰ but a definitive survey of Mexico has not appeared in the indexed literature to date.

Most of the positive reactors were white, although approximately as many Negroes as Caucasians were tested. This finding may be partially explained by the fact that the Negro population in the area surveyed have a tendency not to travel outside of their site of origin. It is of significance to note that all but three of the positive reactors had traveled away from the place of study, some quite widely.

The ages of the positive reactors ranged between 18 and 40, and most of the subjects fell in the age group of 18 to 20. This cannot, however, be said to demonstrate any significant relationship between age and incidence of coccidioidin positivity for this series.

SUMMARY

1. The literature pertinent to the cutaneous test with coccidioidin is reviewed.

2. A report is made of a group of 413 subjects in Dallas, Texas, comprising nurses, medical students, and general hospital patients who were subjected to intracutaneous coccidioidin tests. In this group, an incidence of 2.66 per cent positive reactors was encountered.

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METHOD OF ASSAYING STEROIDS AND ADRENAL EXTRACTS FOR PROTECTIVE ACTION AGAINST TOXIC MATERIAL (TYPHOID VACCINE)

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THE protective action of adrenal extract against the acute toxic effects of typhoid vaccine and the chronic toxic effects of *Staphylococcus aureus* vaccine in adrenal deficient rats was reported by Hartman and Scott¹ in 1932. The results of Ettelson² confirmed their protective power against typhoid vaccine intoxication and demonstrated the relative ineffectiveness of desoxycorticosterone acetate. The possibility of using these observations as an assay method for determining what factor or factors in adrenal extract are responsible for protection against toxic materials was evident. The following method has been evolved and appears to give constant and reproducible results.

METHOD

Male rats (Sprague-Dawley strain) weighing between 80 and 120 grams each were adrenalectomized under ether anesthesia, and after an interval of at least five days the animals were ready for the test. The diet consisted of Purina dog chow. Nine-tenth per cent sodium chloride was offered for drinking. Room temperature was maintained between 80 and 85° Fahrenheit.

The minimum lethal dose (M. L. D.) of typhoid vaccine (Lederle combined TAB vaccine), which would cause 100 per cent mortality within twenty-four hours when administered intraperitoneally, was determined on some of the adrenalectomized rats maintained on 0.9 per cent sodium chloride. The test dose adopted was 1.33 M. L. D. The adrenalectomized animals not used for estimation of the M. L. D. were used for testing the protective power of the material to be assayed. Extracts were administered subcutaneously at 9:00 A.M. and 5:00 P.M. if in aqueous or alcohol-aqueous solution, once a day at 9:00 A.M. if in oil. They were given for three days. Immediately after the last morning injection of extract, 1.33 M. L. D. of typhoid vaccine were administered intraperitoneally. The percentage of animals surviving twenty-four hours later was recorded. The surviving animals were then placed on plain drinking water. Those alive after twenty-one days were sacrificed and searched for remnants of adrenal tissue. If any adrenal tissue was found, the observation on the animal was not used in calculating the protective power of the extract.

One toxic protection unit is defined as the amount of material, in terms of any convenient unit, per twenty-four hours which will protect 90 per cent of adrenalectomized rats against 1.33 M. L. D. of typhoid vaccine, equivalent in protective power to 0.28 mg. 11-dehydrocorticosterone acetate.

RESULTS

The percentage of rats surviving twenty-four hours after 1.33 M. L. D. typhoid vaccine intraperitoneally, when treated with various amounts of adrenal extract, is shown in Fig. 1. When 1 c.c. per twenty-four hours of whole adrenal extract (Upjohn) was given, 90 per cent of the animals were protected; this showed that the extract, according to the foregoing definition, contained 1 toxic protection unit per cubic centimeter.

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The protective power of the extract was nearly completely destroyed when the extract was alkalinized to pH 11.0, boiled for ten minutes, and the pH readjusted to pH 7.0. The equivalent of 2 c.c. of extract after this treatment showed no protective effect. One cubic centimeter of untreated extract protected 90 per cent of the animals.

The potencies of four extracts were determined by the toxic protection test and compared with assay values estimated by the Ingle muscle-work test. They showed 1.0, 2.5, 2.2, and 10.0 toxic protection units per cubic centimeter, respectively, as compared with 1.0, 2.8, 5.0, and 11.5 units by the muscle-work test. One unit by the Ingle muscle-work test is equivalent to the effect produced by 0.2 mg. 11-dehydro-17 hydroxycorticosterone.

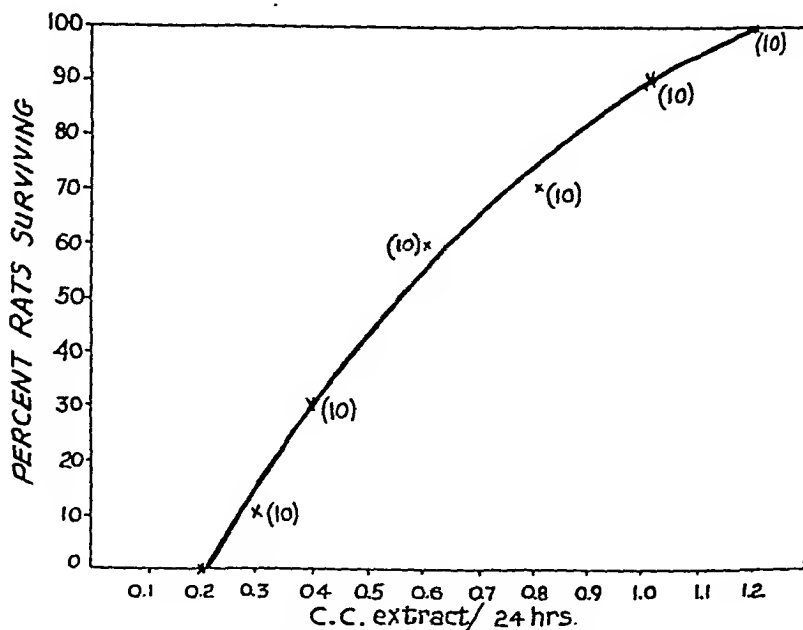


Fig. 1.—Protective power of whole adrenal extract (Upjohn) against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

When the rats were injected with between 3 and 10 mg. of desoxycorticosterone acetate per day, 75 to 85 per cent survived. Summarized in Fig. 2 are the results obtained when desoxycorticosterone acetate was administered once a day in peanut oil. When it was dissolved in propylene glycol and injected twice daily, the protective action at the lower dose levels was essentially the same as that when dissolved in oil. When 5.0 mg. or more, involving the injection of 0.5 c.c. or more of propylene glycol, were given, less protection was observed than when the material was in oil solution.

Free desoxycorticosterone when administered in oil showed a protective power of 1 toxic protection unit per 2 milligrams. The protection of 90 per cent of the animals with 2 mg. of free desoxycorticosterone per day and 100 per cent of the animals with 3 mg. was interesting, since not more than 85 per cent of

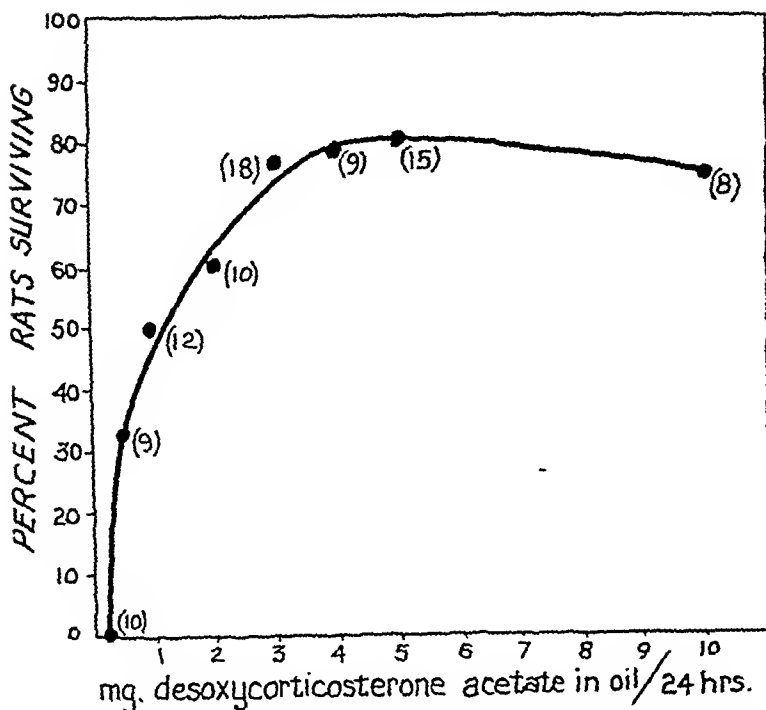


Fig. 2.—Protective power of desoxycorticosterone acetate in peanut oil against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

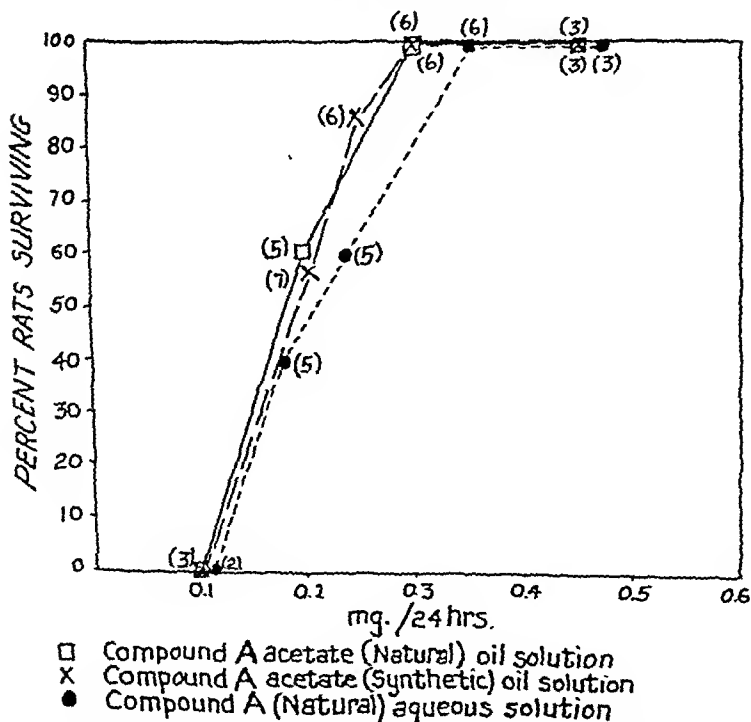


Fig. 3.—Protective power of compound A and compound A acetate against 1.33 M. L. D. typhoid vaccine in adrenalectomized rats.

the animals were protected when the acetate was employed in doses from 2.5 to 10 mg. per twenty-four hours. The slightly greater solubility of desoxycorticosterone over desoxycorticosterone acetate may account for the greater protective action.

Corticenebenzoate protected 100 per cent of the test animals when administered in amounts of 0.75 mg. per day, indicating slightly more than 1.3 toxic protection units per milligram.

A series of experiments was carried out using 11-dehydrocorticosterone and 11-dehydrocorticosterone acetate prepared from adrenal tissue and 11-dehydrocorticosterone acetate synthetically prepared. The protective power of natural and synthetic compound A acetate in oil was essentially the same (Fig. 3), being 3.6 units per milligram. Compound A in aqueous solution administered twice daily showed a protective power of 3.1 units per milligram. The more constant level of the compound in the blood obtained when the hormone was given in oil probably accounts for the apparently greater potency of the compound A acetate. When administered in doses of 3.0 mg. per day, 11-ketoprogesterone protected 100 per cent of the animals treated; while 0.3 mg. per day protected 54 per cent. Progesterone in the same amounts gave no protection.

TABLE I

COMPOUND	TOXIC PROTECTION (UNITS PER MG.)
11-dehydro-17 hydroxycorticosterone	5.5
11-dehydrocorticosterone acetate (natural)	3.6
11-dehydrocorticosterone acetate (synthetic)	3.6
11-dehydrocorticosterone (natural)	3.1
Corticenebenzoate	1.3
11-ketoprogesterone	More than 0.5, less than 3.0
Desoxycorticosterone	0.5
Desoxycorticosterone acetate	Less than 0.1
Acetoxypregnenolone	Less than 0.1
Methyl androstenediol	Less than 0.1
Progesterone	Less than 0.3

Acetoxypregnenolone and methyl androstenediol in doses as large as 10 mg. per day failed to show any protection.

Compound E, 11-dehydro-17 hydroxycorticosterone, showed 5.5 toxic protection units per milligram. This was a little more potent than any other compound tested.

In an attempt to determine whether the maintenance of a normal blood glucose level was the effective mechanism of protection against the typhoid toxin, some of the adrenalectomized animals suitable for testing were injected subcutaneously with 2 c.c. of 5 per cent glucose a half hour before the administration of 1.33 M. L. D. of typhoid toxin. They were injected with a similar amount of glucose solution every two hours or each hour thereafter. Half of the animals, when they began to become moribund, were bled for blood glucose determination. The remainder were kept for the lethal studies. All animals were dead within eight hours following the administration of toxin. The blood glucose levels determined on animals receiving injections each two hours were

70, 95, 65, 105, and 88 mg. per 100 c.c., respectively; the blood glucose levels of those receiving hourly injections were 65, 105, 80, 120, 70 mg. per 100 cubic centimeters; respectively.

DISCUSSION

The protective power of adrenal extract, 11-dehydrocorticosterone, 11-dehydrocorticosterone acetate (natural) 11-dehydrocorticosterone acetate synthetic, corticosterone, 11-dehydro-17 hydroxycorticosterone, and desoxycorticosterone against the toxic effects of typhoid vaccine when administered to adrenalectomized rats closely paralleled their relative potencies as judged by their effects on carbohydrate metabolism. The fact that 11-ketoprogesterone showed toxic protective power was interesting. Data on its effect on carbohydrate metabolism will be published later. All of the compounds with an oxygen at C-11 showed high toxic protection power. The maintenance of normal or near normal blood glucose levels by injection at frequent intervals of 5 per cent glucose in saline was not, however, effective alone in prolonging the life of the typhoid vaccine injected animals. It appears that some other factors besides normal blood glucose must be active in protecting against the typhoid vaccine.

These results suggest the possible usefulness of adrenal extract and certain crystalline preparations of adrenal origin in combating acute toxic reactions. It is possible that the administration of large amounts of these substances would protect patients during the most acute phases of a toxic disease, as in typhoid or typhus fever, especially until the amount of hormone produced by the patient's own adrenal would be adequate for protection.

Further animal experiments are being carried out to determine whether the compounds that have been found effective against typhoid toxin are also able to protect against other types of bacterial toxin.

SUMMARY

A method has been described for determining the relative protective power of adrenal extracts and steroids against the toxic effects of typhoid vaccine on adrenalectomized rats. Of eleven crystalline compounds studied 11-dehydro-17 hydroxycorticosterone was the most potent. All of those compounds having an oxygen at C-11 exhibited marked power to protect against typhoid vaccine. The natural and synthetically prepared 11-dehydrocorticosterone acetate (compound A acetate) showed identical potency.

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TRIDIONE: A NEW ANTICONVULSANT DRUG

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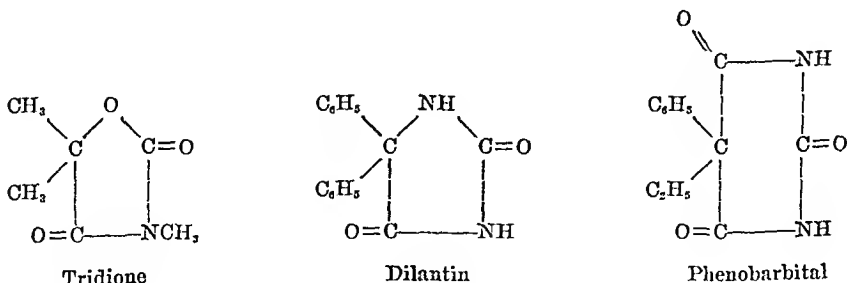
TRIDIONE (3,5,5-trimethyloxazolidine-2,4-dione) was first synthesized by Spielman¹ in a systematic search for new analgesic drugs. Pharmacologic studies indicated that the compound possessed analgesic properties,² and this was later substantiated by clinical investigation. During further studies in animals it was found that Tridione suppressed convulsions caused by other drugs in doses which produced little or no neurologic deficits. This led to a more extensive study of the anticonvulsant properties of the drug by Richards and Everett^{2, 3} who suggested its possible use in epilepsy. Subsequently, Goodman and associates^{4, 5} confirmed and extended the experimental work. Thorne⁶ found this drug effective against grand mal, and Richards and Perlstein⁷ were the first to report its particular usefulness in petit mal. Lennox^{8, 9} has reported extensive clinical studies with tridione.

Clinical experience thus far indicates that tridione is an important addition in the treatment of the petit mal triad, while its possible usefulness in other forms of epilepsy is still controversial and in need of further clarification. However, the marked effectiveness of tridione by intravenous injection is of interest in the management of status epilepticus and convulsions due to various causes. These considerations, as well as the unusual combination of both analgesic and anticonvulsant properties in one drug, justify a description of its pharmacology with special reference to therapeutic applications.

Tridione is a colorless solid with a low melting point (approximately 46° C.). It is soluble in water to 5 per cent and forms a neutral solution. In Table I are given the structural formulas of three synthetic organic drugs used in the treatment of epilepsy, namely, phenobarbital, Dilantin (diphenylhydantoin sodium), and tridione. While each compound is derived from a different cyclic

structure, a common feature is the group $\begin{array}{c} | \\ -\text{C}-\text{C}-\text{N}-\text{C}- \\ | \quad || \quad | \quad || \\ \quad \text{O} \quad \quad \text{O} \end{array}$.

TABLE I



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GENERAL PHARMACOLOGY

One of the outstanding properties of tridione is its low toxicity. The general symptomatology and toxicology is presented in a condensed form in Table II.

Sleep occurs only with very high doses, approaching lethal levels, unless the drug is injected intravenously. With lower doses the animals behave either normally or show various degrees of ataxia. If extremely high doses are given, death occurs as a result of acute respiratory and circulatory depression or after one or two days in coma. A comparison of the action in different species of animals shows qualitatively similar effects and also a rather uniform quantitative sensitivity to the drug. The lethal dose is approximately 1.5 to 2.0 Gm. per kilogram by intravenous, intraperitoneal, or subcutaneous administration, and there is also relatively little difference in the amounts necessary to induce ataxia by various routes of administration. An ataxia-producing dose given orally to mice shows its effects in two or three minutes; tying of the pylorus delays the action for about twenty-five minutes, but the drug is slowly absorbed from the stomach. Only intravenous injection produces short sleep at a dose level which was not soporific by other routes. These observations suggest that tridione is readily absorbed.

Rats fed a diet containing 0.25 per cent tridione showed no effect upon their growth rate, while concentrations of 0.5 or 1.0 per cent retarded weight gain by 15 and 40 per cent, respectively. These feeding experiments were continued for more than nine months. The red blood count of the group fed the highest concentration of the drug was found to be at the lower limit of

TABLE II. EFFECT OF TRIDIONE IN EXPERIMENTAL ANIMALS

GM./ KG.	MICE			RATS	RABBITS		CATS	Dogs
	I.V.	I.P.	ORAL	S.C.	I.V.	I.P.	I.P.	I.V.
0.25		No effect		Slight ataxia		Slight ataxia		
0.30							Slight ataxia	
0.35					Sedation	Slight ataxia		
0.40								Ataxia
0.50	Slight ataxia	Slight ataxia	Slight ataxia	Ataxia	Sedation	Ataxia		
0.60								Sleep (2 min.)
0.75	Ataxia	Ataxia	Ataxia		Sleep	Ataxia		
1.00	Light sleep	Marked ataxia	Light sleep	Marked ataxia		Marked ataxia	Sedation	Sleep (15 min.)
1.50	Sleep	Sleep	Sleep	Sleep (4 to 6 hr.)	LD ₅₀	LD ₅₀		
2.00	Fatal LD ₅₀	Sleep	Sleep and death	LD ₅₀			Prolonged sleep and death	
3.00	Acute death							

normal, while in the other groups no indication of an effect upon the hematopoietic system was noted. Urinalyses disclosed no abnormalities. Histologic sections of organs of animals that died or were killed showed some cloudy swelling of the kidney and liver and occasionally some fat in the latter organ. None of the findings was indicative of any serious organ damage.

The effect upon blood pressure and respiration was studied in dogs and cats under pentobarbital anesthesia. The rapid intravenous injection of 25 mg. of tridione per kilogram produced no effect, but 50 mg. per kilogram produced transitory depression of the blood pressure (30 to 50 mm. Hg). No significant change in respiration was seen. Etherized rabbits tolerated the slow intravenous injection of as much as 50 mg. per kilogram without respiratory depression, while 75 mg. per kilogram produced a marked depression of blood pressure and respiration. Both functions, however, gradually returned to normal.

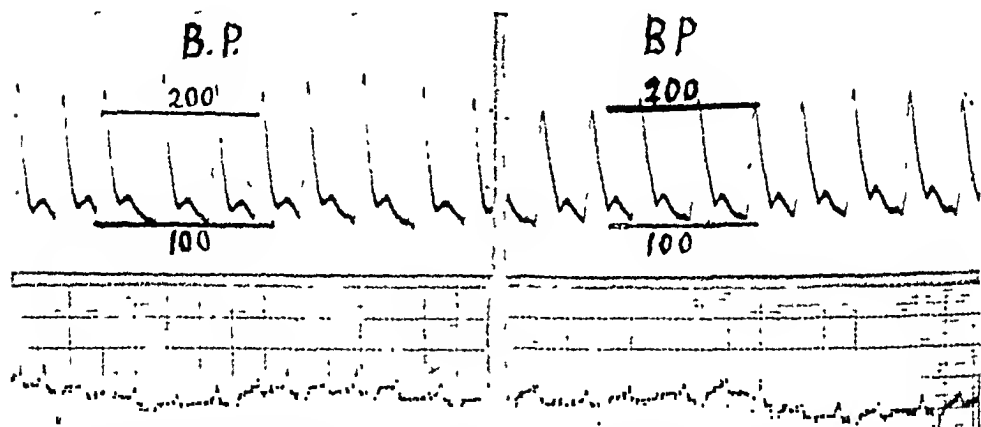


Fig. 1.—Intravenous injection of tridione in a dog weighing 16 kilograms. Upper tracings, blood pressure; lower tracing, ECG Lead II. Left before and right after a total of 3 Gm. of tridione. See text.

The action on the cardiovascular system in unanesthetized dogs was studied by simultaneous recording of the blood pressure and electrocardiograph. Blood pressure was recorded by means of a Hamilton membrane manometer. In Fig. 1 are shown tracings of a dog weighing 16 kilograms taken before the administration of the drug and after three intravenous injections, each of 1.0 Gm. of tridione given at intervals of about four minutes. Two minutes after the last dose the systolic blood pressure was transitorily reduced by approximately 15 mm., while the diastolic reading remained unchanged. No significant change in pulse rate, electrocardiogram, or respiration occurred. Observation failed to detect any sedation, and there was but slight ataxia of the hind legs. Other studies on the blood pressure and on the intestine failed to show any specific effect of tridione upon the reaction to parasympathetic stimulation by acetylcholine. Nor did the drug exhibit any significant antispasmodic action. Effects upon temperature regulation and metabolism have not yet been adequately studied.

The analgesic action of tridione was first reported by Richards and Everett,² and a more detailed account of the findings in experimental animals together with clinical data was given by Richards, Everett, and Pickrell.¹⁰ The question of its practical usefulness in the clinic has not yet been settled. Certain side effects which will be discussed later appear to limit its administration as a routine analgesic to the ambulatory patient. Scrutiny of available reports and our own experience indicate that further study of the use of this drug, particularly in dysmenorrhea and for postoperative pain, is worth while, especially in view of the absence of undesirable effects upon blood pressure, respiration, and the digestive tract.

ANTICONVULSANT ACTION

The anticonvulsive action of tridione in experimental animals has been demonstrated against pentamethylenetetrazol (Metrazol) and other convulsant drugs.³ Recently we have made use of the observation by Walker and co-workers¹¹ that intracortical administration of penicillin produces violent and prolonged convulsions. We found tridione effective in suppressing these seizures in rabbits and dogs. Goodman and associates¹² have reported carefully controlled experiments in which they investigated the effect of tridione and other anticonvulsant drugs upon electrically induced convulsions. Tridione was found to modify and decrease the response to electric shock and had a moderate effect upon the electroshock threshold. It was particularly effective against metrazol convulsions. Of special interest is the observation that "spike and dome" waves resembling a petit mal electroencephalogram can be produced experimentally in rabbits by subconvulsant intravenous doses of metrazol. Tridione was found to be the most effective drug in inhibiting this phenomenon; phenobarbital was less active, and dilantin was ineffective.

The duration of the anticonvulsant action was studied in rabbits. The intravenous injection of 17.5 mg. of metrazol per kilogram produced violent convulsions in each of sixteen animals so treated. If 100 mg. of tridione per kilogram were injected intravenously, ten minutes prior to the metrazol, only 30 per cent of the rabbits convulsed. In another series a forty-five minute interval was allowed between the administration of the tridione and the metrazol; convulsions occurred in 80 per cent of the animals. With a dose of 200 mg. of tridione per kilogram, only 20 per cent of the animals had convulsions when metrazol was given one hour later. These findings are in fairly good agreement with results in mice reported earlier by us.³ It must be kept in mind that the dose of the convulsant agent was very high. Goodman and associates¹² found tridione to be protective against three to five times the convulsant dose of metrazol, depending on the species of animal. Goodman and Manuel⁴ noted a residual anticonvulsive effect against the standard convulsive dose of metrazol, forty-eight hours after the intraperitoneal injection of high doses of tridione.

Information on the rate of urinary excretion of tridione was obtained in three adult men, each of whom took 2.0 Gm. of the drug in a single dose. Urine was collected at two-hour intervals during an eight-hour period. The

urine was extracted with ether, and the residue obtained after evaporation of the ether fraction was titrated with NaOH. The total amount of the drug recovered after eight hours was 47.2, 53.4, and 46.7 mg., respectively. The greater part was excreted within two to six hours after ingestion. This finding shows that only traces of therapeutic doses appear in the urine, and we must assume that the major portion of this drug is metabolized.

The role of the kidneys and liver in the metabolism of tridione was studied in nephrectomized animals and also after liver function was damaged by injection of carbon tetrachloride. If mice subjected to one or the other of these procedures were given 500 mg. of tridione per kilogram orally, no prolonged protection against the convulsive effect of 125 mg. of metrazol per kilogram was noticed in animals with damaged livers and only a slight prolongation was noticed in the nephrectomized mice. From a similar experiment in rats, it was observed that the hypnotic effect of a sublethal dose was prolonged in nephrectomized animals for twenty-four to forty-eight hours. Ataxic dosages gave more variable results but also indicated a slower disappearance of the drug. These observations indicate that therapeutic doses can apparently be destroyed in the body, even in the presence of impairment of the functions of the liver and kidney. With large doses of the drug, however, the importance of these organs becomes more prominent. Further studies are necessary to assay quantitatively the role of liver and kidneys.

As mentioned previously, the clinical usefulness of tridione in the treatment of petit mal epilepsy has been established by Richards and Perlstein,⁷ by Lennox,^{8,9} and by Perlstein and Andelman,¹⁴ and many other investigators are in general agreement. DeJong¹⁵ found tridione effective in combination with other drugs in the treatment of psychomotor attacks. While favorable results in this disorder were also observed by Perlstein and Andelman,¹⁴ more extensive investigation in this condition is clearly indicated. The same applies to its application in the management of disturbances of behavior in children. The need for further study with respect to the usefulness of tridione against grand mal epilepsy has been pointed out. Lennox⁹ indicated that in certain patients suffering from grand and petit mal epilepsy tridione tends to increase grand mal attacks. Perlstein and Andelman¹⁴ prefer a combination with phenobarbital, rather than dilantin, for such patients. Dramatic results in the treatment of a few cases of status epilepticus by intravenous injection of tridione have been reported by Thorne⁶ and by Erickson and Masten.¹⁶ The anticonvulsant action in patients with tetanus is reported by Perlstein and Andelman.¹⁴

The effect of tridione upon the electroencephalogram in petit mal does not appear to be an immediate one in all instances. Gibbs¹⁷ has observed that several hours elapsed before an effect upon the electroencephalogram in certain cases is noted, even after intravenous administration.

SIDE EFFECTS

Minor side effects, such as drowsiness, nausea, and slight skin rashes, have been reported. Of interest is the attempt to overcome the drowsiness which

occurs in certain patients with large doses of tridione by the simultaneous administration of desoxyephedrine, a central stimulant with an action similar to that of amphetamine. Further experiments in mice have shown that it is possible to reduce the sedative effect of tridione with desoxyephedrine without significantly interfering with the protection against convulsive doses of metrazol.

Serious skin rashes in symptomatology and course, resembling those occurring after barbiturates and other drugs, have been reported in a few cases. One of us (R. K. R.) observed an adult male patient who had taken phenobarbital for a considerable time for the treatment of grand mal attacks. About ten days after tridione had been added to control petit mal seizures, the patient developed conjunctivitis quickly followed by a maculopapular rash over the whole body and by a high fever. The exanthema was particularly pronounced on the flexor, palmar, and plantar surfaces of the limbs and became exfoliative in certain areas. Macerations on the mucous membrane of the lips and mouth were present. Treatment consisted of withdrawal of both phenobarbital and tridione and administration of intravenous sodium thiosulfate and intramuscular penicillin. The fever abated after about one week, the rash faded slowly, and the patient recovered. Fortunately, severe reactions of this type are rare.

Recently two cases of blood dyscrasias terminating fatally have been reported.^{18, 19} Both presented aplastic anemia and agranulocytosis. In one of these patients another new drug with potentially toxic action was administered simultaneously with tridione. In spite of the rare occurrence of such reactions, frequent blood counts should be made on all patients receiving tridione.

A peculiar side effect of tridione which we encountered early in the clinical study of this drug is the occurrence of an increased sensitivity to light. The subjective description of this sensation is referred to by some patients as "glare" and by others as a more or less marked loss of color vision which sometimes makes the objects appear yellow or covered with snow. This phenomenon is noticed particularly in bright daylight. Adults are more likely to experience this condition than children. Various degrees of visual disturbances occur in up to 75 per cent of adults in some clinical studies. This side effect appears usually within the first few weeks of the medication. Wearing of dark glasses helps considerably, and in some patients this phenomenon becomes less pronounced during continued medication. Withdrawal of the drug because of this side effect has been found necessary only in 10 to 20 per cent of adult patients (almost never in children) and was always followed by a return of normal color perception and light sensitivity. Sections of the optic nerve and retina of rats fed high doses of tridione for several weeks failed to show any organic damage. In accord with this are the negative findings of ophthalmologists on patients with marked visual symptoms. Similar symptoms sometimes occur with other drugs, for example, digitalis (Carroll²⁰).

CONCLUSIONS

From the experiences of the clinical investigators, it appears that tridione will be an effective drug for the majority of patients suffering from petit mal epilepsy. Children are most benefited, since they show the greatest percentage of favorable responses with a minimum of side effects.

In this early stage in the investigation of tridione, it is not possible to make statements regarding its site of action. The origin of petit mal seizures is still obscure, but it is possible that they arise from subcortical centers. Considering the analgesic action of tridione and the role of the thalamus in pain sensation, one is induced to speculate regarding an at least partially subcortical action of tridione. It appears quite likely that further study of the drug may make it a useful tool for research in various neurophysiologic and pathologic problems.

Tridione is now being investigated clinically in various nervous disorders unrelated to epilepsy, but no statements in this respect can be made as yet.

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ACUTE AND CHRONIC TOXICITY STUDIES ON THEOPHYLLINE AMINOISOBUTANOL AND THEOPHYLLINE ETHYLENEDIAMINE

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THEOPHYLLINE derivatives have been used clinically for a number of years with favorable results.¹⁻⁴ In spite of this, the literature contains relatively few data on acute and chronic toxicity in animals. Ludueña⁵ found that the repeated administration of doses equivalent to those used clinically produced no systemic effects in rabbits. He reported, however, that a single dose of theophylline ethylenediamine, approximately thirty times the clinical dose on a basis of body weight, produced irritation in the meninges and kidneys of rabbits.

Since Ludueña has reported this irritant action for one theophylline derivative, it is important that possible toxic effects of any new theophylline derivative be determined before the substance is tried clinically.

It is the purpose of this paper to present results of toxicity studies on two theophylline derivatives, theophylline aminoisobutanol and theophylline ethylenediamine which contain approximately 67 per cent and 75 to 82 per cent, respectively, of theophylline. Theophylline aminoisobutanol has recently been introduced into clinical medicine⁶⁻⁸ under the trade name of Butaphyllamine, and its pharmacologic actions have been summarized by Hansel.⁹ Data on theophylline ethylenediamine (Aminophylline) are included for comparative purposes and because Ludueña's report makes information on effects of prolonged administration in several species desirable.

Acute Toxicity.—The acute intravenous and oral toxicities were determined for theophylline aminoisobutanol and theophylline ethylenediamine. Intravenous toxicity determinations were conducted in rabbits. The drugs were injected into the marginal ear veins as 25 per cent aqueous solutions. Oral toxicities were determined in mice. The theophylline derivatives were administered, as 5 per cent solutions, directly into the stomach through a rigid tube.

Animals were observed for a period of one week following both intravenous and oral administrations, and all deaths occurring within this period were included in mortality curves. LD₅₀ values and standard errors were determined by the method of Miller and Tainter.¹⁰

Results of the investigations in rabbits and mice indicate that the two compounds have similar acute toxicities. Death following administration of either compound was generally preceded by convulsions and usually occurred within ninety minutes, although in a few instances it was delayed for several days.

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Intravenously, the LD_{50} of theophylline aminoisobutanol was found to be 163 ± 5.65 mg. per kilogram and that of theophylline ethylenediamine 150 ± 7.65 . Orally, the LD_{50} values of theophylline aminoisobutanol and theophylline ethylenediamine were 600 ± 34.7 and 540 ± 12.9 mg. per kilogram, respectively.

Chronic Toxicity.—The effects of repeated administration of theophylline aminoisobutanol and theophylline ethylenediamine were investigated in dogs and rats.

Dogs: Eighteen mongrel dogs were fed a diet of Purina Dog Chow and conditioned to the laboratory for a period of two months before starting the experiment. They were then divided into six groups of three dogs each. Two of the groups served as controls, one for intravenous work and one for oral work. One group was administered theophylline aminoisobutanol orally and another theophylline aminoisobutanol intravenously. The other two groups received theophylline ethylenediamine, one by the oral and one by the intravenous route. Both compounds were administered in doses of 20 mg. per kilogram per day, six days a week, for twelve weeks. These doses are equivalent to 1.2 Gm. per day for a man weighing 60 kilograms, and they are about two or three times the usual clinical dose. The drug solutions for parenteral use were prepared fresh daily in pyrogen-free sterile normal saline. Oral doses were administered in hard gelatin capsules.

Hematologic evaluations were conducted on experimental and control animals at weekly intervals throughout the course of the experiment. The evaluations included erythrocyte, reticulocyte, leucocyte, and differential cell counts and hemoglobin determinations. The hemoglobin determinations were made with a Fisher electrohemometer, and hematocrit determinations were conducted according to the Wintrobe technique¹¹ excepting that minimal amounts of a 10 per cent solution of potassium oxalate were added as an anticoagulant.

All dogs were sacrificed at the termination of drug administration. Specimens from the aorta, heart, lung, liver, kidney, spleen, adrenal, stomach, and small intestine were fixed in Bouin's fluid, and paraffin sections were prepared and stained with Delafield's hematoxylin and eosin. The brain meninges were fixed in formaldehyde, and sections were stained by Mallory's connective tissue method.

At the termination of drug administration, seventeen of the eighteen dogs were in excellent physical condition. They either gained in body weight, remained constant, or lost an insignificant amount. One animal developed a severe case of mange one week after starting the daily administration of theophylline aminoisobutanol orally. This dog had lost an equivalent of 20 per cent of its pre-experimental body weight by the end of the experiment; but considerable weight was lost during the predrug period, and its weight changes cannot be attributed to drug administration. It is concluded, therefore, that the daily administration of the two theophylline derivatives did not prove toxic, as measured by changes in body weight and general condition of the experimental animals.

The two theophylline derivatives did not alter the blood pictures of the dogs. Initial and final average values (Table I), which are typical of the

TABLE I. INITIAL AND FINAL AVERAGE BLOOD VALUES WITH STANDARD ERRORS FOR CONTROL AND EXPERIMENTAL DOGS

COMPOUND	ERYTHROCYTES (MILLIONS PER C. MM.)		HEMATOCRIT (PER CENT CELLS)		RETICULOCYTES (PER CENT)		HEMOGLOBIN (GM. PER 100 C.C.)		THOUSANDS PER C. MM.		LEUCOCYTES PER CENT	
	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL
Theophylline aminoiso- butanol	6.36 ± 0.01	6.20 ± 0.30	37.0 ± 3.9	36.0 ± 3.6	0	0	12.6 ± 0.4	13.4 ± 0.4	11.7 ± 1.2	11.2 ± 0.4	70.8 ± 9.7	62.0 ± 6.5
<i>Intravenous Administration</i>												
Theophylline ethylenedi- amino Control	6.35 ± 0.25	6.72 ± 0.51	34.3 ± 1.0	38.0 ± 1.7	0	0	12.4 ± 0.1	13.5 ± 0.6	10.9 ± 0.8	11.4 ± 0.2	69.0 ± 2.5	63.2 ± 8.7
<i>Oral Administration</i>												
Theophylline aminoiso- butanol	5.49 ± 0.16	6.24 ± 0.15	38.6 ± 1.5	36.0 ± 2.4	0.33 ± 0.24	0.23 ± 0.23	11.7 ± 0.9	13.3 ± 0.9	12.2 ± 2.7	12.6 ± 2.3	67.0 ± 3.6	68.7 ± 6.7
Theophylline ethylenedi- amino Control	6.26 ± 0.48	6.98 ± 0.51	37.0 ± 0.6	39.0 ± 1.2	0	0	12.1 ± 0.4	14.3 ± 0.5	15.1 ± 0.2	10.4 ± 0.9	64.0 ± 11.7	76.0 ± 3.0
<i>Oral Administration</i>												
Theophylline aminoiso- butanol	6.75 ± 0.55	7.50 ± 0.36	40.0 ± 2.2	39.0 ± 3.0	0.63 ± 0.01	0.20 ± 0.20	13.4 ± 0.8	14.8 ± 1.2	9.7 ± 0.3	10.0 ± 1.0	69.8 ± 3.6	71.5 ± 3.2
Theophylline ethylenedi- amino Control	6.82 ± 0.12	6.58 ± 0.15	37.0 ± 1.3	40.0 ± 2.5	0.07 ± 0.01	0.23 ± 0.21	13.3 ± 1.3	14.6 ± 0.8	11.1 ± 1.0	12.1 ± 2.0	65.7 ± 2.0	72.3 ± 7.4

weekly values, fall within a normal range, and there is no significant difference in data for the experimental and control groups.

Microscopic examination of tissue sections from sacrificed animals revealed only minimal pathologic changes and these were not significant. Some abnormalities were found in both control and experimental animals, and the changes were of a type frequently encountered in laboratory animals. They included pneumonia in one dog, thinning of the cytoplasm of liver cells in one,

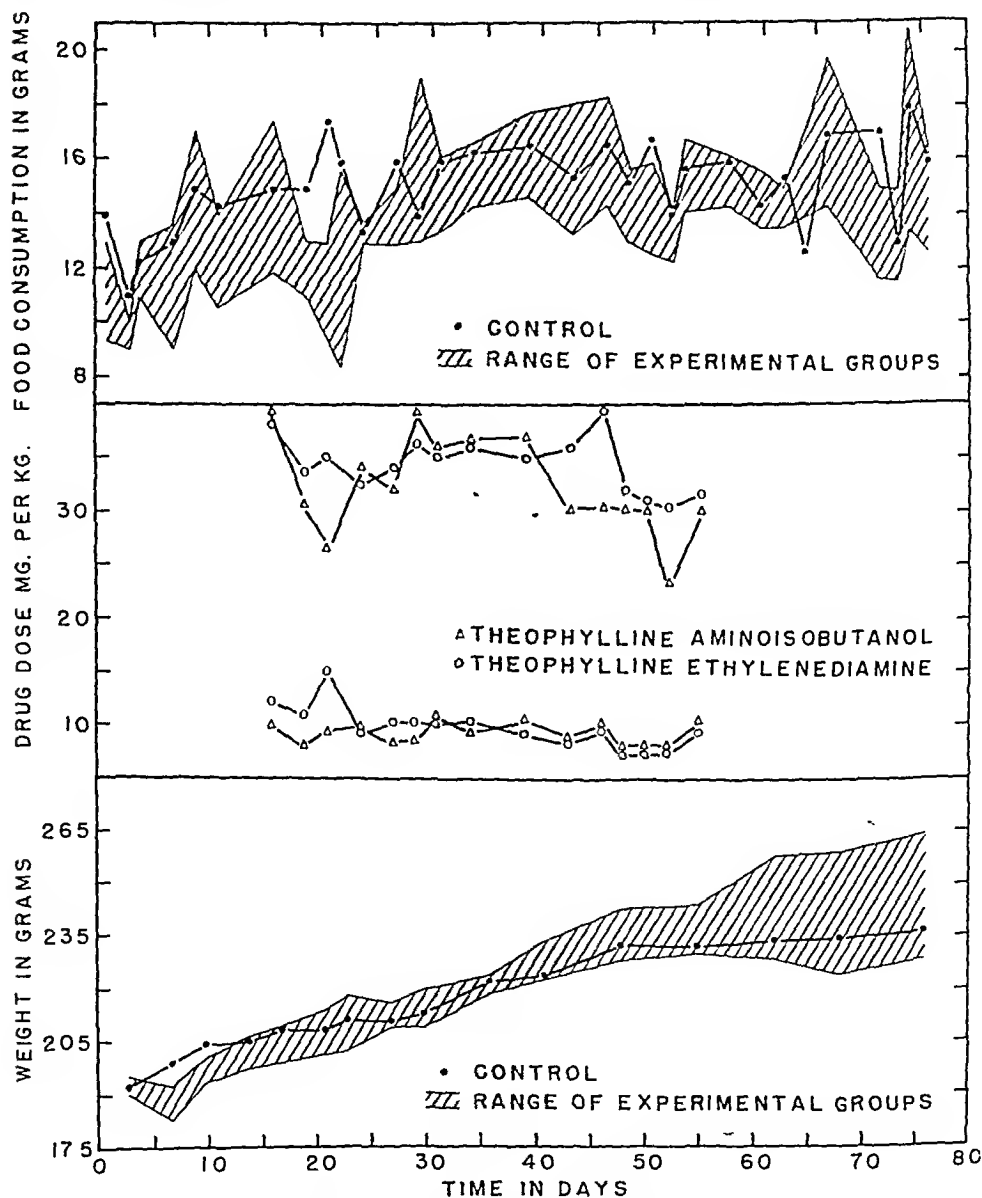


Fig. 1.—Average food consumption, drug dose, and body weight for experimental and control female rats.

TABLE II. INITIAL AND FINAL AVERAGE BLOOD VALUES WITH STANDARD ERRORS FOR CONTROL AND EXPERIMENTAL RATS

COMPOUND	DOSE (MG. PER KG.)	ERYTHROCYTES (MILLIONS PER C. MM.)		RETICULOCYTES (PER CENT)		HEMOGLOBIN (GM. PER 100 C.C.)		LEUCOCYTES			
								THOUSANDS PER C. MM.		GRANULOCYTES PER CENT	
		INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL	INITIAL	FINAL
<i>Female Rats</i>											
Theophylline aminiso- butanol	0	7.83 ± 0.41	8.19 ± 0.33	1.9 ± 0.6	1.2 ± 0.2	15.5 ± 1.1	14.3 ± 0.4	16.4 ± 2.4	15.4 ± 2.0	25.4 ± 3.4	28.4 ± 3.8
	34	7.74 ± 0.26	8.62 ± 0.14	2.0 ± 0.3	1.9 ± 0.3	13.3 ± 0.3	14.7 ± 0.6	12.5 ± 0.2	13.3 ± 0.7	21.1 ± 3.3	26.5 ± 3.3
Theophylline ethylenedi- amine	0	8.30 ± 0.17	8.52 ± 0.13	1.1 ± 0.9	1.4 ± 0.3	14.3 ± 0.7	14.4 ± 0.5	18.9 ± 3.0	15.7 ± 3.4	19.0 ± 1.5	25.1 ± 2.7
	34	7.68 ± 0.30	8.14 ± 0.38	1.7 ± 0.4	1.0 ± 0.1	13.6 ± 0.2	14.0 ± 0.4	16.4 ± 1.6	17.0 ± 1.0	19.6 ± 3.1	26.0 ± 3.6
Control	--	8.11 ± 0.23	8.03 ± 0.36	3.0 ± 0.9	1.9 ± 0.2	14.1 ± 0.4	13.3 ± 0.4	15.7 ± 1.9	16.0 ± 2.1	17.4 ± 2.0	26.0 ± 4.6
<i>Male Rats</i>											
Theophylline aminiso- butanol	0	7.83 ± 0.29	9.35 ± 0.35	3.5 ± 0.3	2.0 ± 0.3	11.6 ± 0.7	13.9 ± 0.6	17.5 ± 1.5	18.5 ± 0.8	26.1 ± 3.1	29.4 ± 2.3
	34	8.41 ± 0.33	9.60 ± 0.29	2.1 ± 0.4	1.9 ± 0.5	14.3 ± 0.6	14.7 ± 0.7	15.7 ± 0.7	21.6 ± 2.8	18.1 ± 1.1	25.8 ± 2.8
Theophylline ethylenedi- amine	0	7.70 ± 0.34	9.28 ± 0.07	4.3 ± 1.5	2.6 ± 0.5	12.5 ± 0.8	14.2 ± 0.8	20.0 ± 2.7	19.7 ± 2.2	22.4 ± 1.7	21.0 ± 2.6
	34	7.91 ± 0.33	9.02 ± 0.25	3.2 ± 0.9	2.7 ± 1.8	13.0 ± 0.4	13.3 ± 0.5	18.4 ± 2.3	22.9 ± 0.3	16.3 ± 1.5	27.3 ± 4.6
Control	--	8.57 ± 0.31	9.80 ± 0.15	2.3 ± 0.4	2.1 ± 0.4	13.9 ± 0.5	15.0 ± 0.5	18.8 ± 1.9	17.8 ± 2.1	19.5 ± 1.6	22.6 ± 5.2

nuclear pyknosis in a few scattered parenchymatous liver cells in two, and minimal amounts of nuclear pyknosis in cells of the nephronic tubules in two animals.

Rats: Five groups of six male and five groups of six female rats of the Wistar strain were employed in evaluating effects of repeated administration of the two derivatives to rats. One group of male and one group of female animals served as controls. The experimental groups received either theophylline aminoisobutanol or theophylline ethylenediamine in doses of approximately 9 and 34 mg., respectively, per kilogram per day by the drug-diet method.

Drug administration was continued for forty days in the female and sixty days in the male animals. One-half of the rats from each group were sacrificed at the termination of drug administration, and the remaining animals were sacrificed three weeks later. Tissues from all experimental and control animals were fixed in Bouin's fluid, and paraffin sections of the aorta, heart, lung, liver, kidney, spleen, adrenal, stomach, and small and large intestines were prepared.

Data in Fig. 1 indicate that the two theophylline derivatives did not alter food consumption or body weight of female animals. Similar results were obtained with male animals; therefore, data for these rats are not presented.

Neither compound caused blood dyscrasia. Erythrocyte, reticulocyte, total and differential leucocyte counts, and hemoglobin concentrations remained in the normal range (Table II), and values for experimental groups did not differ significantly from those of controls.

Microscopic examination of tissue sections revealed no pathologic changes which could be attributed to the theophylline derivatives, either in animals sacrificed at the termination of drug administration or in animals sacrificed three weeks later. The histologic alterations observed were of types commonly found in rats, and they occurred as frequently in control as in experimental groups.

SUMMARY

Theophylline aminoisobutanol and theophylline ethylenediamine were found to have similar LD_{50} values for the oral route in mice and the intravenous route in rabbits, and both compounds produced similar signs of acute toxicity.

The two compounds produced no evidence of toxicity in dogs following the daily administration of 20 mg. per kilogram, either intravenously or orally, and no toxic effects were observed in rats fed amounts of the drugs up to 34 mg. per kilogram daily for as long as sixty days.

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STUDIES ON THE PLASMA CONCENTRATION AND TISSUE DISTRIBUTION OF SODIUM PENTOTHAL (SODIUM ETHYL (1-METHYLBUTYL) THIIOBARBITURATE)

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INTRODUCTION

IN RECENT years sodium pentothal* has become important in anesthesia, especially in the Armed Forces. However, very little is known about its physiologic distribution in the body and its concentration in biologic fluids. The greatest difficulty has been the lack of a specific method for the determination of the drug.

The method commonly employed in the determination of various barbiturates has been the Koppanyi reaction¹ and its modifications.^{2,3} This is a colorimetric method based on the reaction between a barbiturate, cobalt acetate, and isopropyl amine. The reaction is admittedly nonspecific and lacks the sensitivity and accuracy needed for repeated blood level measurements on the same animal.

Anderson and Essex,^{4,5} employing the Koppanyi reaction, studied the plasma levels of pentothal under various conditions and claimed that the drug underwent a cyclic appearance and disappearance in the blood, and there was no correlation between the depth of anesthesia and the blood concentration. This is an unusual phenomenon, for rarely does a drug act in such a cyclic manner.

The purpose of this study was to determine the behavior of pentothal in the blood and tissues after intravenous administration and, if possible, to correlate the depth of anesthesia with the blood level. For such a study a new method of determination was devised which is more specific and more accurate than the Koppanyi reaction.

METHODS AND MATERIALS

Hellman and associates⁶ and Brodie⁷ have studied the ultraviolet absorption curves of pentothal in both organic and aqueous solvents. This is the basis for the present method of determination.

Procedure.—Plasma (0.5 to 1.0 c.c.) and 0.2 M secondary sodium phosphate and 0.1 M citric acid buffer of pH 5 (2 c.c.) are added to redistilled chloroform (7 c.c.) in a small separatory funnel and shaken for three minutes. The two phases are allowed to separate and the chloroform layer is filtered to remove the water droplets. This clear chloroform solution is read in a Beckman quartz

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*Use of the chemical name, sodium ethyl (1-methylbutyl) thiobarbiturate, would be so cumbersome that use of the proprietary designation has been permitted throughout this paper.

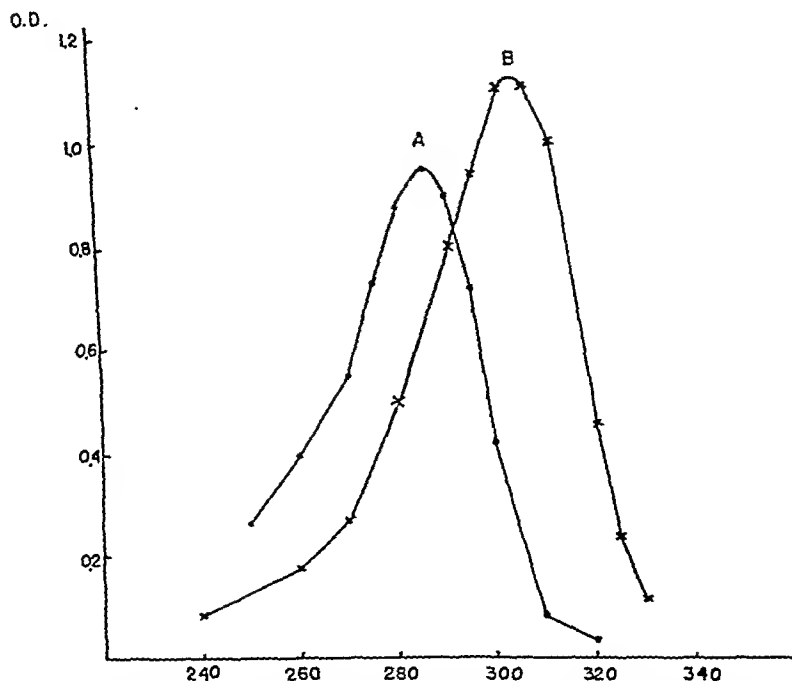


Fig. 1.—Absorption curves of pentothal (A) and sodium pentothal (B) in chloroform and 0.2 N NaOH, respectively. The ordinate represents optical density ($2 - \log. G$) and the abscissa millimicrons.

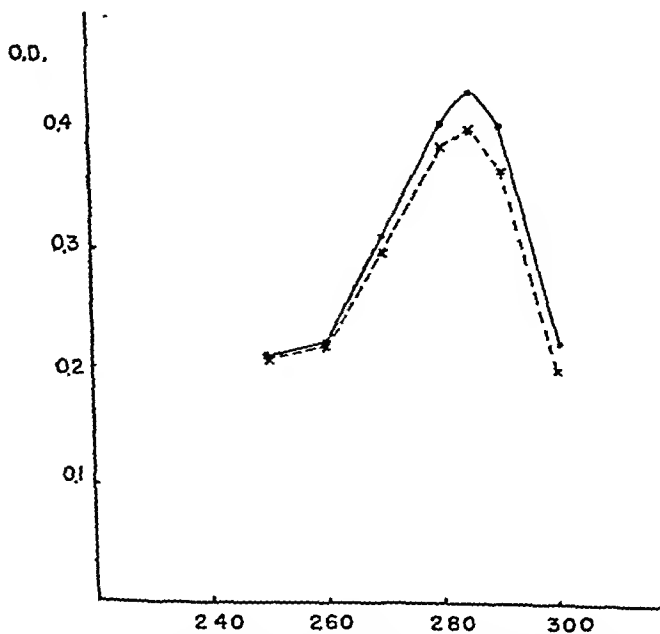


Fig. 2.—Comparison of absorption curves of chloroform extract of rabbit plasma to which pentothal had been added (solid line) and of a similar extract of plasma from a rabbit given sodium pentothal intravenously (broken line).

spectrophotometer at 285 millimicrons. If the chloroform layer is extracted with 0.2 N NaOH and the latter read at 305 $m\mu$, the sensitivity is somewhat increased. The order of magnitude of sensitivity is 0.5 μg per cubic centimeter of the final solution of chloroform or NaOH. Pentothal was prepared by acidifying pentothal sodium and extracting with ether. This was further purified by sublimation under reduced pressure. The resultant pure crystals were used for standards.

The absorption curve of pentothal in chloroform and in 0.2 N NaOH is presented in Fig. 1, and the maximal absorption at 285 and 305 $m\mu$, respectively, is shown. At these wave lengths there is a linear relationship between the concentration and the optical density. Experiments were performed with known amounts of pentothal added to plasma, whole blood, and tissue homogenates, and the recoveries averaged over 90 per cent. In all experiments control plasma was used as a blank (which is small), and known amounts of drug were added to the control in order to determine the percentage recovery (Table I). The specificity of the method is shown in Fig. 2, where the absorption curve of pentothal added to control plasma and extracted with chloroform is compared with that extracted from the plasma of a rabbit which had received the drug intravenously. It is apparent that the materials extracted are identical spectrophotometrically.

In the pharmacologic experiments, five rabbits were slowly injected intravenously with 50 to 100 mg. of sodium pentothal, and blood samples were taken at various intervals by cardiac puncture. In the study of the physiologic disposition of the drug, three rabbits were given 150 mg. and were killed by an air embolus twenty minutes later. The tissue was homogenized in phosphate buffer of pH 5 (0.2 M secondary sodium phosphate and 0.1 M citric

TABLE I

SUBSTANCE	PENTOTHAL ADDED (μg)	PENTOTHAL RECOVERED (μg)	PERCENTAGE RECOVERY
Plasma (c.c.)			
1.0	12.5	12.0	95
1.0	20.0	22.0	108
1.0	20.0	19.0	95
1.0	25.0	23.0	92
Whole Blood (c.c.)			
1.0	10.0	9.9	99
1.0	20.0	19.0	95
1.0	20.0	19.5	98
Liver (Gm.)			
0.5	60.0	58.0	95
1.0	20.0	19.4	96
1.0	20.0	19.6	98
1.5	60.0	56.0	92
Kidney (Gm.)			
1.0	20.0	18.5	92
1.0	20.0	19.0	95
Muscle (Gm.)			
1.0	20.0	19.0	95
1.0	20.0	18.5	92
Brain (Gm.)			
0.5	20.0	19.0	95
1.0	20.0	18.0	90

acid), and the procedure for plasma previously described was followed. Two patients at Walter Reed General Hospital, with only 0.3 mg. of atropine sulfate as preoperative medication, were given pentothal in the usual manner by intermittent intravenous injection in the arm. Blood for plasma levels was taken from the other arm.*

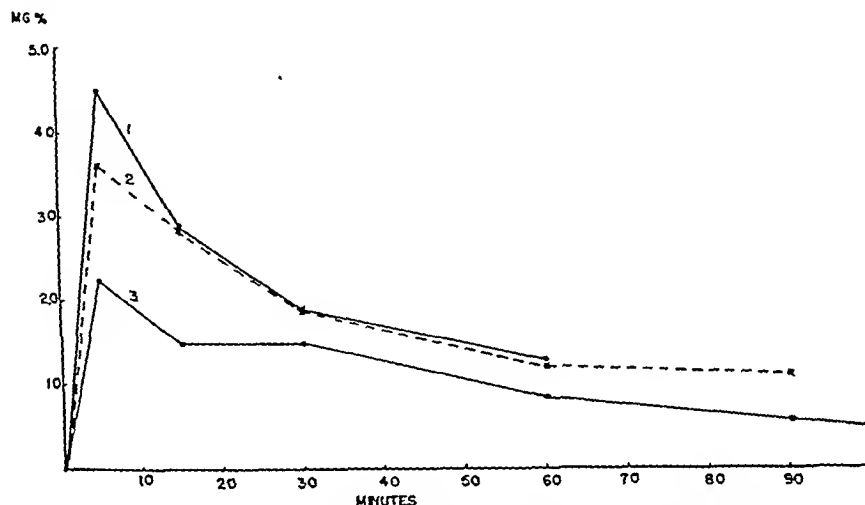


Fig. 3—Plasma concentration after administration of sodium pentothal intravenously. Rabbits 1 and 2 received 100 mg., Rabbit 3, 50 milligrams.

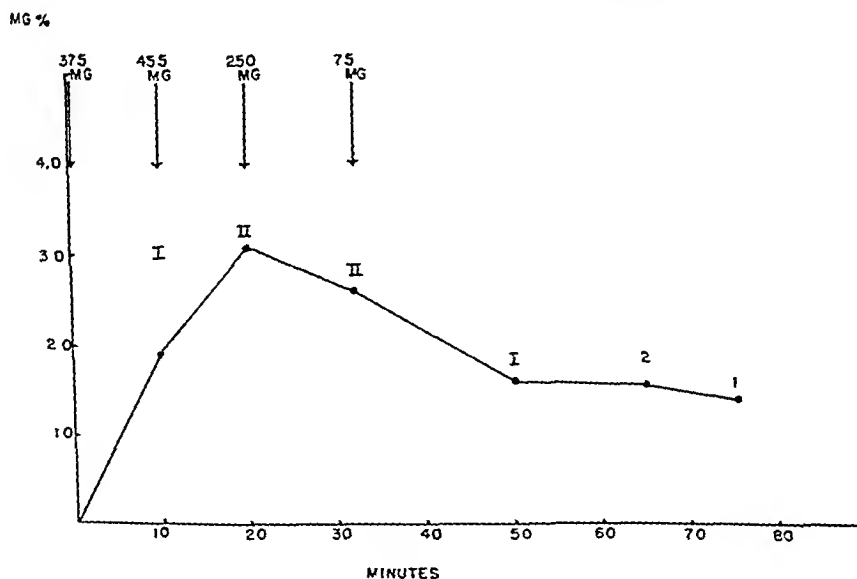


Fig. 4—Plasma concentrations in a human patient during and after administration of sodium pentothal. The arrows show the beginning of the intermittent injections of the indicated amount of pentothal. Those blood samples taken at 10, 20, 32, 59, 65, and 75 minutes following the first dose of drug were withdrawn just prior to administration of the second, third, and fourth doses, respectively. "I" and "II" refer to the plane of surgical anesthesia; "1" and "2" refer to the stage of anesthesia.

*We wish to express our appreciation to Major Emanuel Papper, Chief of the Department of Anesthesia, for doing the clinical aspects of this experiment.

RESULTS

In Fig. 3 is illustrated the course of the plasma level of pentothal after intravenous injection of sodium pentothal in rabbits. The plasma level reaches a peak and falls off rapidly. There is no evidence of any cyclic rise and fall. There seems to be a correlation between the plasma level and the depth of anesthesia. As the anesthesia wears off the plasma level falls. In all cases the concentration of the drug in the plasma was below 1.5 mg. per 100 c.c. when the animal was awake.

Since it is difficult to ascertain the depth of anesthesia in the rabbit, two patients undergoing surgery with pentothal as an anesthetic were studied. In Fig. 4 the stage of anesthesia with the plasma level is correlated. This is merely a preliminary study and more clinical studies are necessary to establish a relationship between concentration and action.

Physiologic Disposition.—The amount of pentothal found in the urine of both rabbits and patients after the administration of pentothal is negligible. Thus, urinary excretion plays a very minor role in the rapid decline of the plasma level. For example, after 1.5 Gm. were given to a patient, only 1.4 mg. were recovered in the urine within a period of two hours.

Whole blood contains approximately the same concentration of pentothal as the plasma, so that it appears that there is no storage of the drug in either the red or the white cells.

TABLE II. TISSUE LEVELS TWENTY MINUTES AFTER INTRAVENOUS ADMINISTRATION OF 150 MG. OF SODIUM PENTOTHAL IN RABBITS WEIGHING 3 KILOGRAMS EACH
(VALUES IN MILLIGRAMS PER 100 C.C. OR 100 GM.)

NO.	PLASMA	WHOLE BLOOD	LIVER	KIDNEY	BRAIN	MUSCLE
1	4.1	3.7	6.9	8.4	2.1	1.9
2	4.6		6.9	12.0	3.1	2.4
3	2.6	2.4	4.3	9.4	1.3	1.0

The distribution of the drug, twenty minutes after the intravenous administration of 150 mg. to a rabbit, is illustrated in Table II. The drug is not stored to any appreciable degree. The kidneys contain more than any other organ, in spite of the fact that so little is excreted in the urine.

DISCUSSION

Ultraviolet spectrophotometry offers a simple, sensitive, specific, and accurate method for the estimation of pentothal in biologic fluids. The substance extracted from the blood after the administration of the drug is identical spectrophotometrically with the crystalline material. Another promising method for the determination of pentothal depends on its fluorescence in strongly alkaline solutions. However, the emitted light has too short a wave length for great sensitivity with the filters at our disposal.

From the data presented, it appears that after the intravenous administration of pentothal the plasma concentration reaches a peak in a short time and falls off rapidly. This decrease is similar to that found with many other drugs, for example, sulfonamides and atabrine. There is no evidence of any

cyclic behavior of disappearance and appearance of the drug in the blood, as has been claimed. There appears to be some correlation between the depth of anesthesia and the concentration of drug in the plasma. Further study of clinical material is necessary to permit an accurate expression of this relationship.

Sodium pentothal is a short-acting barbiturate. Its short action is accompanied by a rapid fall in its plasma concentration after the cessation of the injection of the drug. Negligible amounts are excreted in the urine, and there is very little storage of the drug in the organs of the body. For example, by calculation from Table II twenty minutes after the administration of the drug, only about 20 per cent of the drug administered can be accounted for in the tissues. Therefore, degradation must proceed very rapidly.

SUMMARY

1. A spectrophotometric method for the determination of pentothal has been devised which is simple, accurate, sensitive, and specific.

2. The plasma concentration quickly reaches a maximum level soon after intravenous administration and then falls off rapidly. There seems to be a correlation between the depth of anesthesia and the plasma level.

3. Pentothal is degraded rapidly in the body, since very little is excreted in the urine and there is but a small amount of storage in the organs.

We wish to express our appreciation to Dr. S. G. Hershey for suggesting this problem.

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PYRIBENZAMINE IN HAY FEVER AND OTHER ALLERGIC DISORDERS

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PYRIBENZAMINE is one of a new group of synthetic substances which exert a palliative action on certain allergic manifestations. Experimentally these compounds possess the ability of antagonizing the pharmacologic effects of histamine, which is thought to play some role in the allergic reaction. In comparison to other related compounds the activity of Pyribenzamine is relatively high. Anaphylaxis in laboratory animals is also modified and in some instances prevented by the prior administration of this drug.¹⁻⁵

Pyribenzamine* was administered to over 200 patients manifesting one or more allergic disorders. The drug was supplied by the manufacturer in the form of 50 mg. scored tablets for oral administration. The usual dosage in adults consisted of 50 to 100 mg. four times daily, after meals and at bedtime. In children, 25 to 50 mg. doses were usually employed. Drowsiness, nausea, and vertigo were the most frequent side effects and limited the administration of the drug in many instances.

POLLINOSIS

The effect of pyribenzamine in ninety-eight cases of hay fever was studied. Twenty-eight were due to grass pollen and the remainder to ragweed. The majority of patients had no desensitization treatment to the specific pollen or had received so little that effective results could not be expected from that form of therapy. Some had received adequate pollen treatment but still had symptoms which required attention. For the most part, patients in this group who received no previous desensitization treatment had the severest symptoms.

The evaluation of any therapeutic measure in hay fever must take into account the variation of the daily pollen concentration of the air as well as changing atmospheric conditions. These factors frequently account for increase and decrease in symptoms which can erroneously be attributed to the treatment being administered at those particular times. In addition, patients vary widely in their response to similar amounts of pollen exposure. Some will have minimal symptoms consisting of sniffing and an occasional sneeze, while others will have very marked symptoms such as rhinitis, nasal blocking, episodes of sneezing and conjunctivitis, and irritation of the throat, one or all of which may be present throughout the twenty-four-hour period. The symptoms of many lie between these two extremes. We have classified patients into minimal, mild, and severe, according to the severity of symptoms, in order to better evaluate the effect of pyribenzamine in hay fever.

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*The Pyribenzamine was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Where the action of the drug was favorable, its effect usually became apparent within thirty minutes and lasted several hours. Rhinorrhea and sneezing were more readily alleviated than nasal blocking.

In twenty-eight patients with grass pollinosis (Table I), nineteen received symptomatic relief, while nine showed no satisfactory change. Six of these patients were considered to have had severe symptoms, while twenty-two had mild or minimal difficulty. An appreciable amount of benefit was obtained in the majority of patients in each group.

TABLE I. GRASS HAY FEVER

	MINIMAL SYMPTOMS	MILD SYMPTOMS	SEVERE SYMPTOMS	TOTAL
Improved	8	7	4	19
Not improved	1	6	2	9
Total	9	13	6	28

TABLE II. RAGWEED HAY FEVER

	MINIMAL SYMPTOMS	MILD SYMPTOMS	SEVERE SYMPTOMS	TOTAL
Improved on high and low pollen days	18	7	1	26
Improved on low pollen days only	2	7	11	20
Not improved	5	10	9	24
Total	25	24	21	70

Seventy cases of ragweed hay fever received pyribenzamine (Table II). Attention to pollen counts and comparison with control patients were used in evaluating these results. In many cases symptoms were not completely relieved by the drug; however, where an appreciable improvement was noted, the patient was considered benefited. Twenty-one cases were classified as severe hay fever, twenty-four as mild, and twenty-five as minimal. Since it was found that in the same patient the drug frequently helped on some days, and failed to do so on others, its efficiency was further analyzed according to the atmospheric pollen concentration of that date. Of twenty-one patients with severe hay fever, eleven obtained satisfactory symptomatic relief during periods of low pollen concentration. Only one of the total group, however, obtained discernible benefit when the pollen count was high. In twenty-four patients with mild hay fever, seven were benefited during periods of both high and low pollen counts, while seven others obtained relief on low pollen days only. Eighteen of twenty-five patients with minimal symptoms obtained excellent results during periods of both high and low pollen concentrations. Two other patients in this group noted appreciable benefit only when the pollen count was low. Seven patients with seasonal asthma associated with severe hay fever were encountered during the ragweed season. Pyribenzamine was helpful in relieving asthma in one instance.

OTHER ALLERGIC CONDITIONS (TABLE III)

Nonseasonal Asthma and Allergic Rhinitis.—Thirty patients with non-seasonal asthma received pyribenzamine. Two of these received symptomatic relief whenever the drug was administered. The remainder of the patients did

TABLE III. OTHER ALLERGIC CONDITIONS

CONDITION	TOTAL CASES	HELPED	NOT HELPED
Vasomotor rhinitis	108	69	39
Asthma, nonseasonal	30	2	28
Urticaria and angioneurotic edema			
Acute	9	8	1
Chronic	15	11	4
Eczema			
Atopic	6	5	1
Contact	6	2	4
Pruritis, generalized	8	6	2
Dermatitis, unclassified	4	1	3
Headache allergic	12	0	12
Conjunctivitis, vernal	4	0	4
" " " " "	2	1	1
" " " " "	2	1	1

not experience relief of asthma, although in many cases where allergic rhinitis was also present, a beneficial effect on the nasal symptoms was evident. Of 108 patients with allergic rhinitis, with and without asthma, symptoms were alleviated to some extent by the drug in sixty-nine. The degree of relief in each patient was extremely variable, and just as in seasonal hay fever the symptoms of rhinorrhea and sneezing were more amenable to control than nasal stuffiness. Pyribenzamine appears to be more effective than oral doses of ephedrine in controlling allergic nasal symptoms. In the presence of complicating nasal infection, evidenced by a purulent nasal secretion, the efficacy of pyribenzamine seems diminished. In asthma, ephedrine is still the drug of choice in most cases.

Allergic Skin Disorders.—Pyribenzamine was found very effective in controlling certain cases of urticaria and angioneurotic edema. Eleven patients with chronic urticaria were completely relieved during its administration, while four others failed to attain any appreciable benefit. It was found extremely helpful in controlling symptoms when allergic studies were being carried out in these patients to determine the specific etiologic agent. Eight of nine patients with acute urticaria obtained striking relief of symptoms following the first or second dose of pyribenzamine. The drug was found to exert a rather marked antipruritic action. This property together with the sedative effect frequently accompanying its use were of considerable value in controlling the itching incident to such allergic skin disorders as atopic dermatitis. Pruritis associated with other conditions was also frequently relieved by the drug.

Miscellaneous Allergies.—Twelve patients with headache in whom allergy was considered to play a major role were not helped by pyribenzamine. No benefit was apparent in four cases of vernal conjunctivitis. One of two cases of blepharitis was improved by its use. A patient with gastrointestinal allergy claimed relief of abdominal cramps a short time after a dose of 50 mg. was taken. Another similar case was unaffected by maximum doses.

TOXICITY

Pharmacologic studies in laboratory animals indicate that the toxicity of pyribenzamine is relatively low.⁶ Side effects attending its clinical use in this study occurred in 27.2 per cent of the patients. Very often the same patient

TABLE IV. SIDE EFFECTS

SYMPTOM	NUMBER OF PATIENTS
Gastrointestinal symptoms	19
Drowsiness	18
Vertigo	16
Fatigue	9
Insomnia	6
Nervousness	4
Dryness of mucous membranes	4
Increased nasal congestion	3
Palpitation	2
Headache	2
Numbness of oral mucous membranes	1
Numbness of hands and feet	1
Feeling of tightness in chest	1
Extrasystole	1
Disorientation	1
Dysuria	1
Urticaria	1

experienced more than one undesirable symptom from the drug. The types of symptoms and the frequency with which they occurred are listed in the accompanying chart (Table IV). While gastrointestinal upsets, drowsiness, and vertigo were the most common symptoms, totally different effects such as insomnia, palpitation, and nervousness were occasionally encountered. Three patients experienced increased nasal stuffiness following the use of the drug. Another complained of asthma following each dose, and one patient, who was taking pyribenzamine for symptomatic relief in vasomotor rhinitis with good results, developed urticaria which was found definitely related to the ingestion of the drug. No evidence of chronic toxicity was observed in any of the patients treated.

DISCUSSION

The favorable effect of pyribenzamine in many cases of allergy is in support of the theory that histamine is a factor in these conditions. If histamine is considered to play the principal role in allergic reactions, the ability of pyribenzamine to more readily control certain types of allergy than others must be explained. Factors other than histamine may of course be held accountable. On the other hand, a quantitative difference in histamine release could be responsible for this variability in action. The observations made in cases of hay fever suggest such a possibility. Although approximately 66 per cent of these cases obtained some benefit from the drug, a closer analysis reveals that its effectiveness was most apparent in sufferers with less than severe symptoms, and almost half of these patients obtained relief only during days of low pollen concentration. It is possible that in severe types of hay fever, and in many cases of asthma, the histamine release is excessive and not adequately antagonized by usual doses of pyribenzamine. Larger doses might produce the desired effect in these instances. In the present study, however, the average patient tolerated doses of more than 100 mg. rather poorly.

The action of pyribenzamine is palliative and of relatively brief duration. There is as yet no proof of lasting benefit or permanent cure from its use. It therefore does not eliminate the need for a thorough allergic investigation of

the individual case and for therapy based on immunologic principles where indicated. It is a valuable adjunct to the specific treatment of hay fever, perennial allergic rhinitis, and urticaria.

SUMMARY

1. Pyribenzamine, a drug with specific antihistamine action, was used in various types of allergic disorders. It afforded symptomatic relief in many cases of seasonal hay fever, perennial allergic rhinitis, and urticaria. Its action in asthma was negligible in most instances.

2. Side effects occurred in 27.2 per cent of those patients treated. Drowsiness, gastrointestinal upsets, and vertigo were the most frequent complaints.

3. The action of the drug is only palliative and does not cure the allergic condition. It is a valuable symptomatic drug and is recommended as a helpful adjunct to the specific treatment of allergic disorders.

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LABORATORY METHODS

STUDIES ON STREPTOMYCIN*

III. MICROTECHNIQUES FOR ASSAY

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VARIOUS methods of assaying streptomycin and streptothricin described in the literature are, for the most part, modifications of penicillin assay techniques. Using a sensitive strain of *Bacillus subtilis* as a test organism for streptothricin determinations, Foster and Woodruff^{1,2} employed a modified cylinder-plate method as described by Abraham and associates³ for penicillin determinations. Similarly, Denkelwater, Cook, and Tishler⁴ employed the cup method for assay to determine the degree of inactivation of streptomycin and streptothricin by cysteine. The turbidimetric method, the serial dilution procedure with *Escherichia coli* as the test organism, and the cylinder-plate technique using *B. subtilis* cells or spores were suggested by Waksman, Bugie, and Schatz.⁵ Stebbins and Robinson⁶ proposed a modified agar cup-plate method for assaying streptomycin in body fluids, using a special strain of *Staphylococcus aureus* as a test organism. For estimating the concentration of streptomycin in body fluids, Heilman⁷ employed a technique similar to Fleming's^{8,9} slide-cell method; *Bacillus megatherium* was used as the test organism instead of *Streptococcus pyogenes*. Fleming found it necessary to heat blood for thirty minutes at 50° C. to kill leucocytes when assaying whole blood. Donovick and co-workers¹⁰ recommended a broth dilution method, using a sensitive strain of *Klebsiella pneumoniae* as a test organism. It was found that fluids to be assayed had to be sterile and had to be diluted to contain from 1.0 to 3.0 units of streptomycin per cubic centimeter. Price, Nielsen, and Welch¹¹ suggested a turbidimetric serial dilution method, using *Bacillus circulans* as the test organism, which they found sensitive to 0.15 microgram of streptomycin base per cubic centimeter of fluid. A method for the quantitative determination of streptomycin in aqueous solutions and in various organic solutions by the filter paper disk technique using *B. subtilis* as a test organism was described by Loo and associates.¹² By rigid standardization, Levy Schwed, and Sackett¹³ obtained a polarographic method for assaying solutions of partly purified streptomycin containing 200 units per cubic centimeter or more of the drug. Using a gram-negative rod for a test organism and a modification of the assay method of Stebbins and Robinson,⁶ Smith¹⁴ detected as little as 0.1 per cent

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*Unless otherwise designated, streptomycin as referred to in this paper is streptomycin hydrochloride. One unit of streptomycin as used in this publication is that amount of material which will inhibit the growth of a standard strain of *Escherichia coli* in 1 c.c. of nutrient broth or other suitable medium.

of streptothricin present as a contaminant in streptomycin. While the organism was inhibited by 1,000 or more units of streptomycin, it was inhibited by only 7.5 units of streptothricin.

In a recent publication, Forgaes, Kornegay, and Henley¹⁵ described a pad method of streptomycin assay to be used primarily for oxalated whole blood and other body fluids. Although the method proved to be accurate to 0.5 unit of the drug per cubic centimeter of body fluid, it required 20 c.c. for a complete standard curve and 1 c.c. for a blood level determination when assaying in quintuplet. This made it difficult to follow drug level determinations in infants or in individual small laboratory animals.

Two methods, described in this publication and designated as the micro-method and microfilm method, circumvent this difficulty. Using the micro-method, 1.5 c.c. of blood are required for a complete standard curve (0.5 through 40 units) and 0.06 c.c. for a drug level determination in quintuplet. For a low unitage standard curve (0.5 through 15 units), only 1 c.c. is required. When using the microfilm method of assay, 1 c.c. of body fluid is required for a standard curve (0.1 through 1.0 unit) and the same amount for a drug level determination as required by the micromethod.

EXPERIMENTAL METHODS

Preparation of Spore Suspension.—

Strain: A variant strain of *B. subtilis* Cohn *emend.* Prasmowski, designated in this laboratory as the I strain, was chosen as the standard assay organism for reasons stated in a previous publication.¹⁵ The techniques described therein were used for preparing spores. Since difficulty was experienced by various workers in sporulating this organism when using the medium previously recommended, a further study of the medium is in progress and results will be reported at a later date. Spores used in this investigation, however, were prepared on the medium previously recommended.

Assay Technique for Micromethod.—

Preparation of Seeded Plates: For assaying, 200 c.c. of Difco E-2 Streptothricin Assay Agar were melted, cooled, and maintained at 50° C. in a water bath. Prior to assay, the agar was seeded with *B. subtilis* spores (approximately 400,000 spores per cubic centimeter of agar) and maintained at 50° C. until all air bubbles emerged to the surface. Twenty cubic centimeter portions of inoculated agar were transferred to large flat-bottomed Petri plates (150 by 20 mm.), using a 30 c.c. glass syringe equipped with an automatic syringe filler to facilitate rapid pouring. (For 100 by 10 mm. Petri plates, 10 c.c. of seeded agar were poured per plate.) Seeded plates were allowed to cool and harden for ten minutes at room temperature. They were then dried uncovered at 50° C. for ten minutes to remove condensed moisture and allowed to return to room temperature for ten minutes with covers partly open.

It was found essential that plating be performed on a level table to obtain a uniform depth of agar and that air bubbles disappear from the agar before it hardened. All timing was carefully checked.

Plates prepared for assay could be stored at 2° C. for future use; however, there was a loss of accuracy proportional to length of storage time. Such plates were again dried at 50° C. and allowed to attain room temperature before use.

Addition of Assay Material: Each plate selected for assay was superimposed over an area marked on a level table so that assay drops could be placed in the same relative position on each consecutive plate. Following adjustment of the plunger in a 0.25 c.c. tuberculin syringe to the 0.05 c.c. graduation mark, 0.06 c.c. of fluid to be assayed was drawn up into the syringe. The air space between the plunger and assay material established complete visibility of the plunger tip relative to the 0.01 c.c. graduations on the barrel of the syringe and also facilitated complete removal of assay material from the syringe. A 27 gauge, 1/2 inch needle was placed firmly on the shank of the syringe with the needle's beveled opening down and opposite the graduations on the barrel of the syringe. Precisely 0.01 c.c. of fluid was slowly ejected from the syringe to form a hanging drop on the end of the needle. This drop was touched to the surface of the seeded agar and thus deposited in the appropriate position on the plate. (When the drop was first ejected from the syringe, the instrument had to be held in a horizontal position in order to facilitate adhesion of the drop to the needle.) This procedure was carried out in quintuplet for all samples assayed, one drop on each of five plates. Using this procedure, samples of assay material sufficient to assay in quintuplet could be completed in from ten to fifteen minutes. One individual can successfully complete the operation.

Incubation and Reading of Assay Plates: Assay plates were incubated overnight (approximately seventeen hours) at a temperature ranging from 28 to 30° C., removed from the incubator, and the diameter of the zone of inhibition of growth measured. If desired, results of the assay could be read in eight hours after the material had been added to the plates.* Readings were recorded to the nearest 0.1 millimeter. Values used were within plus or minus 1.0 mm. of one another. For instance, if five readings for any hypothetical value were 21.0, 21.6, 20.9, 21.4, and 22.7, the last value (22.7) was discarded. The average diameter of the zone of inhibition for each level of the standard was plotted on graph paper against units per cubic centimeter for the respective dilutions of the standard to constitute a standard curve (see Figs. 1 and 2). The diameter of the inhibition zone for any unknown assay sample was compared with the standard curve to give results in units of streptomycin per cubic centimeter of fluid.

Any plate-counting apparatus could be used for reading inhibition zones, provided it was equipped with a transparent, graduated film ruled in 1 mm. areas. The diameter could also be read with a millimeter rule. It was found convenient to use a glass plate covered with a positive exposed negative of

*Preliminary results indicated that by incubating the seeded plates for three to six hours at the temperature given, prior to the addition of the assay material, assays could be read in five and two hours, respectively. However, there was a decrease in accuracy directly proportional to time of initial incubation.

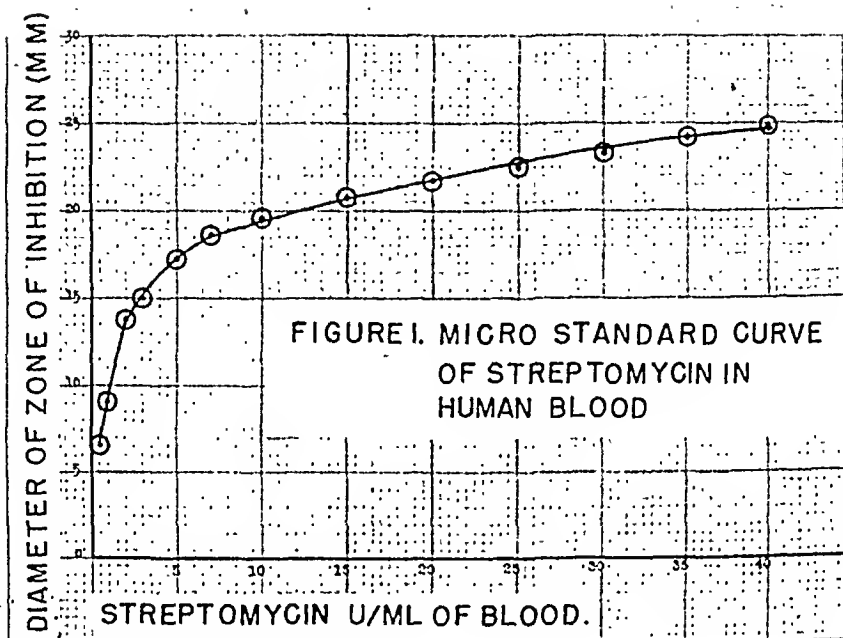


Fig. 1.

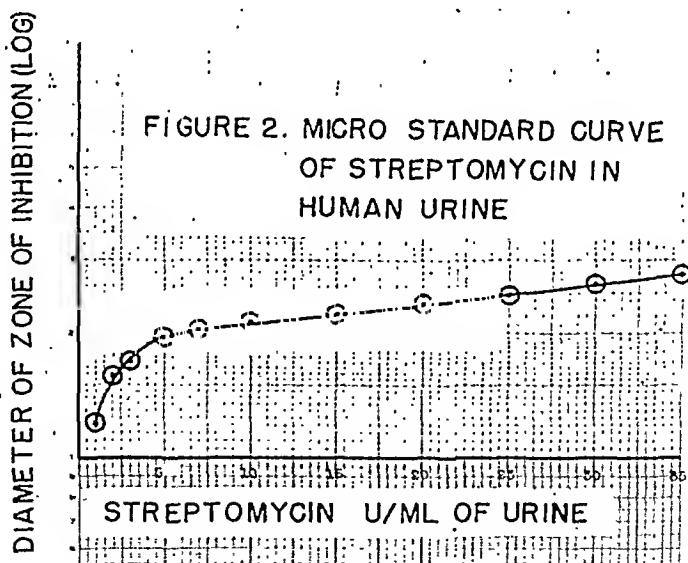


Fig. 2.

1 mm. graph paper on lithographic film.* The diameter of the inhibition zone was superimposed over the plate against a background of light, and the zone thus was read.

*One millimeter graph paper was photographed onto a contrast photographic plate.

Preparation of Standard Curves: Prior to treatment, a 1.5 c.c. sample of body fluid was obtained from the patient for preparing a complete (0 through 40 units) streptomycin standard curve. (Only 1 c.c. need be drawn for a low unitage standard curve, 0.5 through 15 units.) To 0.09 c.c. aliquots of selected fluid, varying concentrations of streptomycin were added and assayed as previously described. (It is imperative that chemically clean 0.1 c.c. serologic pipettes be used for making dilutions and that glassware be thoroughly cleaned each time after use.) The diameter of the resulting zone of inhibition was plotted on graph paper against the corresponding unitage (see Figs. 1 and 2). For example, in following the blood level of streptomycin in a patient, a sample of blood was taken before administration of the drug for constructing a standard curve on blood of that particular person. Blood was collected in lithium oxalate (0.06 c.c. of a 3.3 per cent lithium oxalate solution per 1 c.c. of blood) and mixed. Then 0.09 c.c. of blood was accurately pipetted into each test tube ($\frac{1}{2}$ inch in diameter and 1 inch in height). To each of these, 0.01 c.c. of the respective streptomycin solutions of known variable unitage was added. In pipetting blood and standard solutions, the outer surface of the pipette was wiped to remove excess fluid after it was filled and before delivery to the test tube. Blood was allowed to flow slowly from the pipette into the test tube from the 0.01 c.c. mark to its maximum gravitational level: the remainder was gently blown out and the tip of the pipette touched to the side wall of the test tube. Blood without added streptomycin was assayed to determine possible inhibition of *B. subtilis* by the blood itself. Water standards of streptomycin were prepared so that when 0.01 c.c. was added to 0.09 c.c. of blood or other fluid, concentrations of 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 10.0, and 15.0 units of streptomycin per cubic centimeter resulted for the low unitage curve, and thereafter up through 40 units per cubic centimeter in steps of 5 units for the high unitage curve. (Higher unitages can be approximated.)

Streptomycin standard dilutions of 0.5, 1, 2, 3, 5, 7, 10, and 15 units were placed on one series of five plates and those of 20, 25, 30, 35, and 40 units on another series when using large Petri plates (150 by 20 mm.). When using small Petri plates (100 by 10 mm.) standards from 0 through 10 units were placed on one plate series and standard dilutions from 15 through 30 units on another series of five plates. A third series was used for determining 35 and 40 unit standard dilutions. It is recommended that a complete standard curve be made on each individual prior to initiation of drug therapy.

Manipulation of Body Fluids for Drug-Level Determination.—

Blood: The following technique is recommended to be used in drawing blood for a drug-level determination. Lithium oxalate solution is drawn up into a 0.25 c.c. tuberculin syringe to the 0.15 c.c. mark; the syringe is then inverted (needle end up), and air and remainder of oxalate are extruded, with the exception of that remaining in the shank of the syringe. The residual oxalate solution forms an airtight seal between the plunger and cylinder wall. The plunger is drawn up to the 0.05 c.c. mark. This air space serves as a marker between the blood and plunger, since it leaves the tip of the plunger clearly visible, and also makes it possible to remove all of the blood when assaying.

The finger or other extremity is punctured and 0.06 c.c. of blood *immediately* drawn into the syringe. In drawing blood it is necessary that no air be incorporated into the blood column. This may necessitate squeezing the finger several times until 0.06 c.c. of blood is obtained with no break in the blood column in the syringe. Blood is drawn up into the syringe to the point where the barrel and shank join, being careful that no air is incorporated into the blood now in the syringe. With the shank of the syringe pointing downward, the contents are agitated by gently tapping the side of the cylinder wall with the back of the finger. If the procedure is carefully operated, no air is incorporated into the blood and the oxalate adhering to the syringe wall is adequately mixed with the blood. A 27 gauge, $\frac{1}{2}$ inch needle is tightly placed onto the shank of the syringe with the beveled edge of the needle opposite the graduation marks on the cylinder wall. With the syringe in a horizontal position, the plunger is pushed forward until blood begins to extrude from the needle, and the tip of the plunger is calibrated on a 0.01 c.c. mark. The tip of the needle is then wiped to remove the trace of blood. Then 0.01 c.c. of blood is extruded from the syringe and the tip of the drop touched to the surface of the agar, thus depositing a drop of blood. It has been found that blood thus drawn can be stored in a refrigerator overnight and assayed the following day. This is convenient for determining drug levels over a period of twenty-four hours or longer, in order that blood may be collected at appropriate intervals and assayed at a later time.

TABLE I. VARIATIONS IN INDIVIDUAL BLOOD MICROSTANDARD CURVES

STREPTOMYCIN ADDED (UNITS PER C.C.)	DIAMETER OF ZONE OF INHIBITION (MM.)					
	DOG			HUMAN BEING		
	DOG A	DOG B	DOG C	PATIENT 1	PATIENT 2	PATIENT 3
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	6.9	5.3	8.7	6.4	5.5	0.0
1.0	9.0	7.7	12.4	8.0	7.1	6.2
2.0	13.1	11.7	15.1	12.1	10.3	8.0
3.0	15.0	13.3	16.8	14.8	13.2	10.4
5.0	17.4	16.1	18.9	17.2	15.4	13.6
7.0	18.8	17.3	19.4	19.5	16.6	14.6
10.0	19.6	18.1	20.9	20.5	18.5	17.0
15.0	20.9	19.9	22.4	21.5	20.0	18.1

Data for blood standard curves from 0.5 through 15 units in three dogs and three human beings are presented in Table I to illustrate the assay method and to demonstrate the necessity for individual standard curves. A typical micromethod blood standard curve in human beings is shown in Fig. 1. In Fig. 3 are illustrated inhibition zones on a typical plate when assaying blood by the micromethod.

Urine: Streptomycin levels in urine were determined using, for the most part, the same technique as for blood. However, when assaying urine of high unitage (above 40 units), the specimen was diluted with normal urine to the recommended range of 0.5 to 40 units per cubic centimeter for greater accuracy. In preparing a standard curve, 0.1 c.c. of a sample of streptomycin of known unitage was added to 0.9 c.c. of urine for each respective standard

to minimize errors in pipetting. In addition, it was not necessary to maintain a 0.05 c.c. air pocket between the plunger and urine in the syringe when assaying clear specimens. Any convenient amount of urine can be drawn up into the syringe as long as the specimen is assayed in quintuplet. The semilogarithm standard curve on the urine of the patient as presented in Fig. 2 was used to estimate better the very high drug levels.

Assay Technique for Microfilm Method.—This method, developed by the co-author (J. L. K.), was designated as the microfilm method because it requires only a film of seeded agar and is very sensitive for detecting streptomycin in quantities as low as 0.1 unit per cubic centimeter of body fluid.

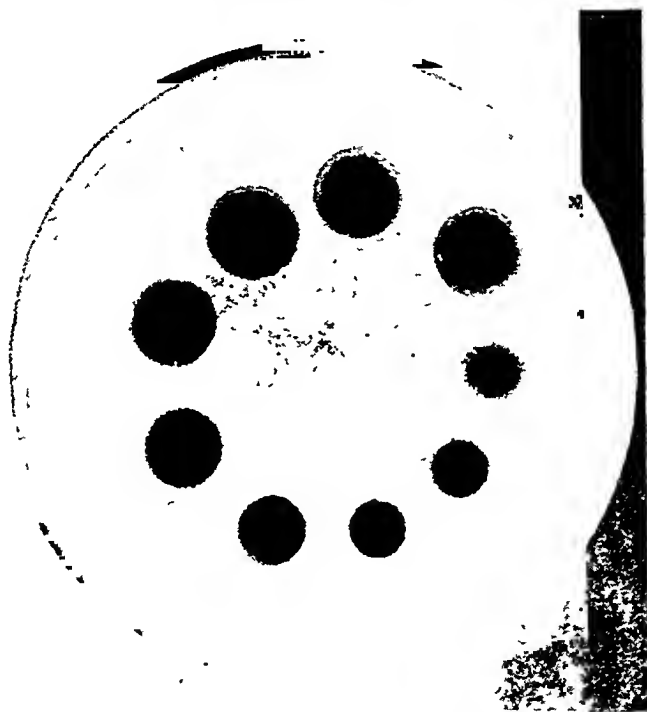


Fig. 3.—Illustrating the type of inhibition zone produced by streptomycin, using the micro-method of assay.

Preparation of Seeded Plates: Difco E-2 Streptothricin Assay Agar was melted, cooled, and maintained at 50° C. in a water bath. It was then seeded with *B. subtilis* spores (approximately 800,000 spores per cubic centimeter of agar), mixed, and maintained in the water bath until the air bubbles emerged from the medium. Approximately 20 c.c. of inoculated agar were added to large sterile Petri plates (150 by 20 mm.). Plates containing the agar were then immediately tipped, and the agar was allowed to pour from the plates into a container for five seconds. The plates were then covered, placed on a flat table, and allowed to harden for ten minutes. This procedure resulted in the formation of a film of agar on the bottom of the plates. The plates were then ready for assaying purposes. Flat-bottomed plates were not necessary when using this technique.

When using small plates (100 by 10 mm.) the same procedure was followed, with the exception that 10 c.c. of seeded agar were added per plate and allowed to flow into a container for the given time (five seconds).

Addition of Assay Material and Incubation and Reading of Assay Plates: The same techniques were used as described for the micromethod.

Preparation of Standard Curves: One cubic centimeter samples of various body fluids have been used for preparing standard curves. The following unitages were used: 0.1, 0.2, 0.3, 0.5, 0.7, 1.0. Although unitages of 2.0, 3.0, and 5.0 could be determined, it was more convenient to assay fluids of such potency by the micromethod. A sample of normal body fluid was assayed to detect possible *B. subtilis* inhibiting factors by the body fluid itself. Techniques and precautions in making standards were the same as for the micromethod; however, the previously designated unitages (0.0 through 1.0 unit) were placed on one plate using either the large (150 by 20 mm.) or small (100 by 10 mm.) Petri plates.

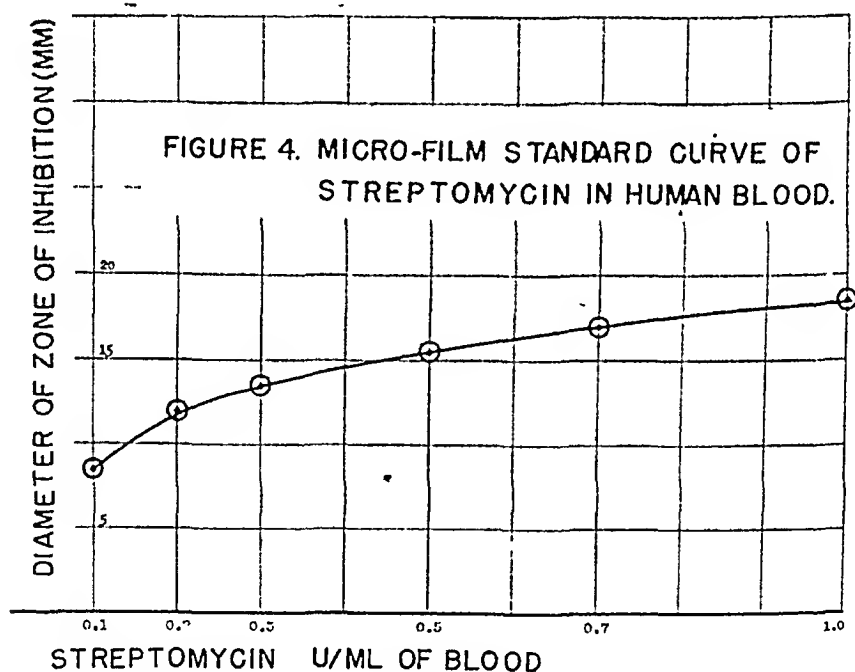


Fig. 1.

In Fig. 4 is illustrated a typical standard curve obtained when using the microfilm method of assaying streptomycin.

Application of Assay Techniques.—Application of these methods for determining streptomycin blood levels in man and in various laboratory animals will be discussed in a succeeding report. The methods are particularly useful where only small amounts of blood can be obtained, such as in mice, guinea pigs, hamsters, and especially in infants where venous puncture is difficult.

In addition, time and material are saved since no pads or cups are required for these methods of assay.

The microfilm assay method was found useful for detecting traces of streptomycin in blood and tissue extracts from animals used for streptomycin excretion studies.

These methods have also been applied successfully to the assay of other antibiotics.

SUMMARY

Two methods, the micro and microfilm techniques for the quantitative determination of streptomycin and other antibiotics in body fluids are described in detail. Using these methods, 1.5 and 1.0 c.c., respectively, of body fluid are necessary for a standard curve and 0.06 c.c. for a level determination in quintuplet. Venous puncture is necessary only for obtaining blood for a standard curve. A drop of blood obtained from a finger or other convenient extremity is used for blood level determinations.

The authors wish to acknowledge their gratitude to Miss Lucy A. Collins, for suggesting development of a micromethod of streptomycin assay, and to Captain Lewis L. Coriell and Dr. Keith H. Lewis, for their suggestions and careful guidance.

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AN IMPROVED PROCEDURE FOR THE DIAGNOSTIC CULTURE OF MAMMALIAN TUBERCLE BACILLI

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WITHIN the past few decades marked strides have been made in the perfection of the diagnostic culture procedure for disclosing the presence of small numbers of mammalian tubercle bacilli. In this short span clinical pathologists and bacteriologists have begun to acknowledge that the culture could efficiently displace the guinea pig or other animal tests¹ for the disclosure of the presence of small numbers of mammalian tubercle bacilli in tuberculous materials from human or animal sources. In spite of the fact that the diagnostic culture procedure is more economical and is superior to the animal test in regard to disclosing the presence of small numbers of moderately or low virulent mammalian tubercle bacilli, even though its superiority is not especially greater for the disclosure of highly virulent human or bovine tubercle bacilli, there are still features of the cultural test which in all probability can be improved materially at present.

The diagnostic culture tests consist of several essential features, the success of the performance of which are significant both in its simplicity and perfection. The first of these features and the part to be particularly elucidated in this study is the preliminary treatment of the specimen to destroy contaminating and undesired microorganisms; while the second equally important feature is the choice of a good nutrient capable of supporting particularly the growth from small plants of mammalian tubercle bacilli.^{2, 3}

Historically, the earliest attempts to destroy the contaminating microorganisms made use of such rapidly acting reagents as sodium hypochlorite (Labarraque's solution) with which Griffith experimented in 1914.⁴ He found that successful uncontaminated cultures of tubercle bacilli would result if plants were made at short minute intervals and the proper time were hit when the reagent would not destroy the tubercle bacilli, but the contaminants were satisfactorily eliminated from the specimen. Shortly thereafter, Petroff⁵ used 3 per cent sodium hydroxide for thirty minutes for the same purpose and with fair success. Later, 3 per cent hydrochloric and 6 per cent sulfuric acid were tried⁶ also with success for short period exposure up to an hour or so. In each case, however, the reagent was prepared from liquid materials of uncertain composition or from solids, such as sodium hydroxide, not entirely easy to handle or weigh and subject to change by absorption of carbon dioxide from the air or capable of etching the glass containers in which they were stored. The need for a crystalline solid of high purity and stable composition was attained with the introduction of 5 per cent oxalic acid in 1930.⁷ This

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reagent was easily prepared and kept well, ready for use. It was found fairly satisfactory in that it could be used in equal volume of 5 per cent solution to destroy contaminants by exposure up to two hours at incubator temperature, giving a greater time latitude, and in many respects it proved superior to sodium hydroxide. The problem of the nutrient for growing small plants of tubercle bacilli resolved itself around two questions: the choice of a good nutrient and essentially the choice of a simply prepared nutrient.

In Dorset's whole egg medium and its modifications,⁸ all the prerequisites for a good nutrient were present, but the possibilities of supporting the growth of small plants remained undisclosed because of lack of adequate test methods in those days. Petroff's gentian violet egg medium contained sufficient retardant dye not only to retard the contaminants but also to retard small plants of tubercle bacilli; and Corper's potato cylinder medium,⁹ though a suitable nutrient, was too easily prepared improperly by the technician to become generally popular. Thus, also a number of the more complicated multiple mixture mediums, like Petragnani's, Loewenstein's, Evanoff and Sweany's, Sasano and Medlar's, and other mediums, proved not entirely satisfying because of complications in preparation. Among the simply prepared mediums, the glycerol egg yolk medium¹⁰ when properly prepared appeared to offer simplicity, ease of preparation, and a good nutrient to recommend it; however, it is still open to improvement of certain features such as overcoming the seasonal variation of eggs or the use of improperly stored fresh eggs with the consequent detrimental effect of spoilage products on the growth of the bacilli. All in all, however, the egg yolk medium properly prepared and from fresh eggs proved the simplest and most efficient of nutrient mediums thus far recommended for the growth from small plants of mammalian tubercle bacilli. The matter of coloring the medium for contrast discernment of early colonies was left to the individual choice of the technician, since the retardant effect on contaminants was found to be unimportant and the color was merely added as an inert stain for contrast. As a matter of fact, experienced technicians preferred to use the egg yolk without color and were able to note early colonies just as easily. However, for those desiring a color background, the color became a matter of individual choice covering a wide range from red through green, blue, and even black. The only drawback to dark colors was that a deeply colored egg medium coated the inside of the tube at the observation zone many times and thus defeated the very purpose for which it was added. It was for this reason probably that many technicians chose malachite green or congo red, dyes without intense colors. When analyzed, however, the color matter is not of great significance in the method or efficiency of the nutrient so long as it possesses no retarding action on the growth of small plantings of mammalian tubercle bacilli.¹¹

Destroying contaminants or preventing their multiplication during storage of the specimen before adding the reagent for destroying them, however, is of most importance to the ultimate success of the cultural procedure, since it becomes more difficult to clean out contaminations the more heavily the specimen is seeded with them.

With the hope of finding a less active or potent crystalline material obtainable in pure form, which might allow more time for preparation of the specimen and which might even be added to the specimen immediately on collection, a number of chemicals were given consideration for the purpose of replacing the commonly used sodium hydroxide (3 per cent) and oxalic acid (5 per cent) reagents. After consideration of a large number of chemicals in the past, a chemically pure trisodium phosphate used in crude form in laboratories for glassware cleaning purposes was given serious consideration. In preliminary tests it was found that a 10 per cent concentration (equivalent to 23 per cent of the chemically pure hydrated salt $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) added to equal volumes of fine suspensions of mammalian tubercle bacilli proved not only innocuous to the bacilli but was more protective than suspension of the bacilli in 0.9 per cent saline solution for long periods of time. The effects of 3 per cent sodium hydroxide, 5 per cent oxalic acid, and 10 per cent trisodium phosphate on suspensions of tubercle bacilli compared with saline suspensions for intervals up to two weeks of contact are recorded in Table I.

TABLE I. EFFECT OF 3 PER CENT SODIUM HYDROXIDE, 5 PER CENT OXALIC ACID, AND 10 PER CENT TRISODIUM PHOSPHATE ON GROWTH OF TUBERCLE BACILLI

TIME OF EXPOSURE TO REAGENT AT 37° C.	REAGENT											
	SALINE			3% SODIUM HYDROXIDE			5% OXALIC ACID			10% TRISODIUM PHOSPHATE		
	AMOUNT OF TUBERCLE BACILLI IN MILLIGRAMS PER CUBIC CENTIMETER* TREATED											
	1.0	10-3	10-6	1.0	10-3	10-6	1.0	10-3	10-6	1.0	10-3	10-6
0	2†	2	3	2	2	51‡	2	2	4	2	2	3
2 hr.	2	2	3	2	3	0	2	3	61	2	2	3
4 hr.	2	2	3	2	3	0	2	3	0	2	2	3
8 hr.	2	2	3	2	3	61	2	4	0	2	2	3
1 day	2	2	3	2	3	0	3	0	0	2	2	3
2 days	2	2	3	2	3	0	0	0	0	2	2	3
4 days	2	2	52	2	4	0	0	0	0	2	2	4
1 wk.	2	42	0	2	42	0	0	0	0	2	3	4
2 wk.	2	0	0	4	0	0	0	0	0	2	4	6

Note: At one week, 10 per cent trisodium phosphate prevents death of bacilli which normally takes place in saline suspensions.

*To 1.0, 0.001, and 0.000,001 mg. per cubic centimeter of tubercle bacilli in fine suspension was added an equal volume of the reagent. After the incubation period indicated, the NaOH and Na_3PO_4 solutions were neutralized with 5 per cent HCl, and the oxalic acid was neutralized with 3 per cent NaOH before planting. The plantings are necessarily one-half of those indicated.

†The time of contact for killing contaminants in sputum and other specimens with 3 per cent NaOH and 5 per cent oxalic acid is thirty minutes to one hour at 37° Centigrade.

‡The numeral indicates the number of weeks after which growth first appeared; the exponent indicates the number of tubes positive when all three tubes planted did not reveal growth.

It is noted from the data recorded in Table I that the addition of the 10 per cent trisodium phosphate in equal volume to suspensions of tubercle bacilli is less detrimental over a period of two weeks at 37° C. than control saline suspension. Equal volumes of both 3 per cent sodium hydroxide and 5 per cent oxalic acid show detrimental effects which become evident after two hours of contact at 37° C.; this effect is more pronounced for oxalic acid after one day than for sodium hydroxide, although the latter is equally harmful to small numbers of bacilli after as early as two hours' exposure.

To note further the effect of an equal volume of the 10 per cent trisodium phosphate at room temperature (20 to 25° C.) as compared with incubator temperature (37° C.) on tubercle bacilli and the contaminants in positive sputums, six of these sputums were tested following contact with the reagent at intervals up to one week's time. The results of these findings are recorded briefly in Table II.

The data recorded in Table II indicate that the trisodium phosphate in equal volumes of a 10 per cent solution (23 per cent of the hydrated salt $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) destroys the contaminants in these positive sputums on exposure for one or two days at room temperature and within about one day at incubator temperature (37° C.); whereas, even one week's exposure at incubator temperature (37° C.) was not particularly detrimental to the viability of the tubercle bacilli, although the longer interval of one week's contact retards their growth.

In order to note further the advantages or disadvantages of neutralizing or washing the treated suspensions, the experiment recorded in Table III on positive sputums was performed.

From the results recorded in Table III it is to be noted that either neutralization or washing out the phosphate produces more satisfactory results when compared with the planting in the phosphate as such. When the phosphate is neutralized or washed out, neither the room temperature nor the incubator temperature appear to be especially detrimental, although the incubator temperature is slightly more so. However, it is significant that the bacilli in sputum can be kept in the phosphate mixture for up to one week without preventing subsequent recovery of the tubercle bacilli planted on a good nutrient medium such as the glycerol egg yolk medium.

The superiority of the one-day treatment at 37° C. with an equal volume of 10 per cent trisodium phosphate compared with the standard one-hour treatment at 37° C. with an equal volume of 5 per cent oxalic acid is illustrated from the examination of eighteen microscopically negative smear and guinea pig test negative specimens of sputum from custodial cases. These disclosed three positive findings for one highly virulent and two moderately virulent strains of human tubercle bacilli as presented with the data recorded in Table IV.

Human feces are difficult to prepare satisfactorily for cultural purposes, and Table V presents a comparison of oxalic acid (5 per cent) and sodium hydroxide (3 per cent), with different time exposures from one day to one week at 37° C., to the trisodium phosphate (10 per cent).

It is noted from the data recorded in Table V that while both oxalic acid and trisodium phosphate are more efficient in clearing up the contaminants in feces than is the sodium hydroxide, the trisodium phosphate proved superior to both the oxalic acid and sodium hydroxide in that a one-week exposure at 37° C. succeeded in a number of cases in removing the contaminants from the human feces. However, no satisfactory method has been found for recovering tubercle bacilli from feces, and this phase will be reported on fully in a subsequent detailed paper.

TABLE II. EFFECT OF 10 PER CENT TRISODIUM PHOSPHATE* ON CONTAMINANTS AND ISOLATION OF MAMMALIAN TUBERCLE BACILLI FROM POSITIVE SPUTUMS

TIME OF CONTACT WITH REAGENT ^m	ROOM TEMPERATURE										INCUBATOR TEMPERATURE (37° C.)									
	NUMBER DESIGNATION OF SPUTUM																			
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6		
+*	C†	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C	+	C	
1 day	3†	3	0	3	0	3	1	3	3	③	3	2	3	0	3	0	3	1	3	0
2 days	3	1	3	0	3	1	3	1	3	1	3	1	4	0	3	1	3	0	3	1
4 days	3	0	3	0	3	0	3	0	3	0	3	1	4	1	4	0	3	0	3	0
1 wk.	3	0	3	0	3	0	3	0	3	0	3	2	7	0	4	0	3	0	3	0

Note: Ten per cent trisodium phosphate (Na_3PO_4) is equivalent to 23 per cent trisodium phosphate weighed with the water of crystallization ($\text{Na}_3\text{PO}_4 \cdot 12 \text{H}_2\text{O}$).

Note: Ten per cent trisodium phosphate (Na_2PO_4) is equivalent to 23 per cent trisodium phosphate weighed with the water of crystallization ($\text{Na}_2\text{PO}_4 \cdot 12 \text{H}_2\text{O}$).

*A, Cultures positive for tubercle bacilli.

†C, Contaminations of three tubes planted.

‡The number of weeks after which growth of tubercle bacilli first appeared.

§A O around a number means contaminated after the culture had been positive for tubercle bacilli.

TABLE III. POSITIVE CULTURES AND CONTAMINATIONS RESULTING FROM TRISODIUM PHOSPHATE TREATMENT OF POSITIVE SPUTUMS FOLLOWING NEUTRALIZATION OR WASHING OF TREATMENT MIXTURE BEFORE PLANTING

TIME OF EXPOSURE TO REAGENT	ROOM TEMPERATURE										INCUBATOR TEMPERATURE (37° C.)									
	SPUTUM NUMBER																			
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
A†	B†	C†	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1 day	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†
2 days	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†
4 days	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†
1 wk.	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†	3†

*A, Not neutralized.

†B, Neutralized.

‡C, Washed, not neutralized.

§Number, Weeks in which positive culture first appeared; exponent, number of tubes of three which were contaminated.

TABLE IV. ISOLATION OF MAMMALIAN TUBERCLE BACILLI FROM MICROSCOPIC SMEAR AND GUINEA PIG NEGATIVE SPUTUMS

PATIENT	RESULTS OF CULTURE TEST		VIRULENCE TEST WITH BACILLI ISOLATED ¹²
	5% OXALIC ACID TREATMENT (1 HOUR AT 37° C.)	10% TRISODIUM PHOSPHATE TREATMENT (1 DAY AT 37° C.)	
W. E.	0	0	
S. H.	0	0	
A. B.	0	0	
G. U.	0	+	Moderately virulent
G. R.	0	0	
H. A.	0	0	
C. O.	0	+	Highly virulent
R. I.	0	0	
T. O.	0	0	
E. K.	0	0	
K. E.	0	+	Moderately virulent
B. O.	0	0	
R. A.	0	0	
S. E.	0	0	
B. A.	0	0	
R. O.	0	0	
M. I.	0	0	
O. Y.	0	0	

Note: We are indebted to Dr. Fritz Rosenberg, Medical Director of the Ex-Patients' Tubercular Home of Denver, for these sputums which were obtained from old custodial cases.

TABLE V. EFFECT OF DIFFERENT PREPARATION REAGENTS ON CONTAMINANT MICROORGANISMS IN HUMAN FECES OBTAINED FROM PATIENTS WITH POSITIVE SPUTUMS

FECES NUMBER	REAGENTS USED TO DESTROY MICROORGANISMS				
	2 VOL. 5% OXALIC ACID TO 1 VOL. FECES FOR 1½ HR. AT 37° C.	2 VOL. 3% SODIUM HYDROXIDE TO 1 VOL. FECES FOR 1½ HR. AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 1 DAY AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 3 DAYS AT 37° C.	2.5 VOL. 10% TRISODIUM PHOSPHATE TO 1 VOL. FECES FOR 1 WK. AT 37° C.
9646	4*	4	3	2	0
9391	1	4	4	4	2
9590	2	4	4	4	0
9537	1	4	4	4	1
9507	4	4	4	4	2
9529	3	4	4	4	1

*The number of contaminated tubes of 4 planted with neutralized treated feces.

TABLE VI. EFFECT OF SODIUM HYDROXIDE, OXALIC ACID, AND TRISODIUM PHOSPHATE TREATMENT UPON MOLDS AND ACID-FAST SAPROPHYTE

MICROORGANISM	REAGENT	TIME OF CONTACT AT 37° C. (HR.)	TEMPERATURE OF CULTURE TEST FOR VIABILITY	
			ROOM	37° C.
Penicillium notatum	3% sodium hydroxide	1	+	0†
		2	0	0
	5% Oxalic acid	4	-	-
Actinomyces griseus	10% Trisodium phosphate	1	+	0
		24	0	0
	3% NaOH	2	-	+
Acid-fast saprophyte (Day)	5% Oxalic acid	1	0	0
	10% Trisodium phosphate	24	-	+
	10% Trisodium phosphate	1½	0	0

*Growth of the test mold or acid-fast saprophyte.

†Absence of growth

In order to note the effect of sodium hydroxide, oxalic acid, and trisodium phosphate on *Penicillium notatum* and *Actinomyces griseus*, as well as on an acid-fast saprophyte, the experiment recorded in Table VI was performed.

The findings recorded in Table VI indicate that the trisodium phosphate (10 per cent) is capable of destroying *P. notatum* within one hour at 37° C. and twenty-four hours at room temperature, and sodium hydroxide (3 per cent) does this within one hour at 37° C. and two hours at room temperature; however, oxalic acid (5 per cent) applied for four hours at 37° C. is unable to destroy this organism. With *A. griseus* just the reverse is true in that oxalic acid (5 per cent) is capable of destroying this organism in one hour at room temperature. Sodium hydroxide (3 per cent) for two hours at 37° C. and trisodium phosphate (10 per cent) for one day at 37° C. are inactive. Acid-fast saprophyte (Day) is destroyed by one and one-half hours' contact with 10 per cent trisodium phosphate. These findings suggest that it may be necessary at times to use both alkali (trisodium phosphate) and acid (oxalic acid) treatment to destroy certain mold contaminants, if we can draw conclusions from the tests with *P. notatum* and *A. griseus*.

SUMMARY AND CONCLUSIONS

1. A new reagent, trisodium phosphate, has been tested to determine its value in comparison with the standard reagents, including oxalic acid and sodium hydroxide, for destroying contaminants in tuberculous materials in preparation for the cultural diagnoses of tuberculosis and for isolating mammalian tubercle bacilli.

2. Trisodium phosphate is a reagent which is a pure crystalline chemical material of standard composition. In 10 per cent solution (23 per cent of the $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), it can remain in contact with tubercle bacilli for up to a week at room temperature without destroying small numbers of tubercle bacilli, but it destroys the undesirable contaminants in sputum within twenty-four hours at 37° C. or within several days to a week at room temperature.

3. Trisodium phosphate as a reagent for destroying contaminations found in tuberculous materials can also be placed in the receptacles used for collecting the tuberculous specimen and thus immediately prevents the development of molds and undesired contaminants. This procedure assures better materials for culturing mammalian tubercle bacilli and therefore the loss of valuable specimens.

4. The time required for destroying contaminants by the trisodium phosphate is one day at 37° Centigrade. This also adds to the convenience with which the technician can prepare his specimens for culture, since there is no necessity for haste in the preparations such as is required with the previously used reagents sodium hydroxide and oxalic acid.

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MODIFICATION OF THE FAUST METHOD IN THE DETECTION OF CYSTS AND OVA

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THE Faust method for the concentration and flotation of cysts and ova has proved valuable as a diagnostic aid in certain infections of the gastrointestinal tract, particularly amebiasis.¹ During the summer and fall of 1945, ever-increasing troops with amebiasis were arriving from the Pacific Theatre of Operations. The diagnosis on some of these cases was confirmed immediately by the direct smear method alone due to the great number of *Endamoeba histolytica* cysts present. Nevertheless, some clinically diagnosed cases continued to exist in which negative results were consistently obtained both by the direct smear and the Faust zinc sulfate flotation methods. Experience had taught us that the latter is the better method in the search for cysts, so we began to suspect that our negative results occurred because cysts were inconsistently present in the stools or because the clinical diagnosis was incorrect. As recommended by Faust and others,¹ patients were purged with a saline cathartic and subsequent stools were examined.² Both vegetative and cyst forms were recovered in some of the cases, the latter forms being found mostly by the zinc sulfate method. In spite of the purgation, only a few cysts were found. The remaining negatives were examined daily for a week and remained negative even after purgation. Thinking that in the routine method of Faust the stool sample might be too small, we decided to modify the method by combining it with my method for detecting schistosoma which had given excellent results in over 25,000 examinations of 800 patients with schistosomiasis japonica.³ The modified method is as follows.

THE MODIFICATION

1. Place about 10 Gm. of feces in a 125 c.c. Erlenmeyer flask containing about 75 c.c. of warm water (about 40° C.); stopper the flask and shake until emulsified.

2. Strain through 1 layer of wet gauze into a 50 c.c. centrifuge tube (teated bottom).

3. Centrifuge for one minute at 2,500 r.p.m. and pour off supernatant fluid. Add warm water to the sediment from a regulated faucet (force of the water will disperse the fecal particles and cleanse them) and centrifuge again at the same time and speed. Decant supernatant fluid; repeat one more washing and centrifugation; again decant supernatant fluid.

4. Add about 10 c.c. of warm water and agitate sediment by shaking; then pour quickly all of contents into a 15 c.c. centrifuge tube. Centrifuge for one minute at 2,500 r.p.m. and decant supernatant fluid.

5. Add about 10 c.c. of zinc sulfate solution (33 per cent, specific gravity 1.180) to the sediment and stir with applicator sticks. Add zinc sulfate nearly to top of tube. Centrifuge for one minute at 2,500 r.p.m. Ova and cysts will float to the surface.

6. Take several loopfuls (baeteriologic loop) from surface and place on slide; add 1 drop of D'Antoni's or Lugol's iodine and cover with a cover slip. Examine for ova of helminths and cysts of protozoa under low-power objective of microscope.

DISCUSSION OF METHOD

This method varies from the original method (Faust's) as follows:

1. A much larger sample of feces is utilized, thereby increasing possibility of detection in lightly infested cases or when cysts and ova are rare in the stool. Cysts and ova quite often are not evenly distributed and a large sample is desirable. Cysts, particularly, may exist in "packets" and can be missed from a remote small section. If only one cyst or ovum is present on a slide (this quite often occurs), the average technician may experience great difficulty in finding it.

2. The use of warm water (about 40° C.) certainly has advantages over the use of regular or so-called cold water. If cold water is used, washing of the sediment can be accomplished and most of the bile coloring matter is removed; yet, this type of washing is not complete because a loose scum remains with the sediment and has a tendency to float when the zinc sulfate is added, thereby impairing the clear field which should exist for the microscopic examination. The so-called fatty type of stool offers practically the same difficulty, and the use of cold water is not of too much value. On the other hand, the use of warm water eliminates a high percentage of the scum or fats. After the water is added the scum rises to the surface within one minute, remains floating after the centrifugation, and eventually is discarded with the supernatant fluid. Each additional washing brings up less scum until it is practically all discarded. When the zinc sulfate now is added, the surface of the liquid is fairly clean and makes a good field for microscopic examination. Some workers have suggested the use of cold physiologic saline for the washing, but this is also inferior to plain warm water.

3. The initial use of the large 50 c.c. tube instead of the smaller test tube is first of all important because it is necessary to receive a large sample from the original 10 Gm. of stool. Our purpose would be defeated if 10 Gm. of stool were originally emulsified and only 5 to 10 c.c. of the emulsified fluid examined. With the teated bottom of the 50 c.c. tube there is less tendency for cysts or ova to be lost in the decanting process. With the larger tube the proportion of sediment to the size of the tube decreases the number of washings and centrifugations in arriving at a clear supernatant fluid; three or four washings usually give this desired result and a clean zinc sulfate preparation.

CONCLUSIONS

A modification of the Faust method is described, which increases the yield of cysts and ova as well as *Strongyloides larvae*. It is of particular value when cysts, ova, and larvae are rare in the fecal specimens.

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A RAPID PYRIDINE SILVER STAIN FOR NERVOUS TISSUE AND RETICULAR FIBERS

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THE explosive nature of ammoniacal silver solutions¹⁻³ and the technical difficulties attendant upon their use for staining nervous tissue elements and reticular fibers have led to the development of a simple and rapid silver stain for these purposes. Pyridine is employed instead of ammonium hydroxide in the preparation of the silver stain, rendering the solution easy to prepare and obviating the danger of explosions. For routine purposes the method is useful for demonstrating axons and dendrites of neurons, Alzheimer's neurofibrillary change, fibrillary astrocytes, and reticular fibers.

I. To Demonstrate Axons, Dendrites, and Alzheimer's Neurofibrillary Change.—

1. Collect formalin-fixed frozen sections about 25 μ each in distilled water containing a trace of ammonium hydroxide.

2. Transfer sections to silver pyridine solution for about six to ten minutes. Solution is prepared by mixing the following together until clear:

Silver nitrate, 1 per cent	2 c.c.
Potassium carbonate (anhydrous), 1 per cent	1.5 c.c.
Pyridine (pure)	0.16 c.c.

3. Transfer sections directly to excess 10 per cent formalin neutralized with calcium carbonate for about one minute.

4. Float sections onto slides, dehydrate through alcohols and xylols in usual manner, and mount in balsam. Axons, dendrites, Alzheimer's pathologic neurofibrils, and nuclei stain black against yellow background.

II. To Demonstrate Fibrillary Astrocytes.—The method is exactly the same as for nerve cell fibers, except that fixation is carried out in formalin bromide (2 Gm. of ammonium bromide in 100 c.c. of 10 per cent formalin). Processes of fibrillary astrocytes stain black, while axons and dendrites are usually poorly stained.

III. To Demonstrate Reticular Fibers.—

1. Hydrate routine formalin-fixed paraffin sections in usual manner.

2. Transfer slides to potassium permanganate $\frac{1}{4}$ per cent solution for five minutes. Rinse in tap or distilled water.

3. Transfer to oxalic acid 5 per cent solution for five minutes. Rinse well in distilled water.

The views expressed in this paper are those of the author and should not be construed as necessarily reflecting the views of the Navy Department.
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4. Transfer to silver pyridine solution, prepared by mixing the following together successively until clear, and stain for seven minutes:

Silver nitrate, 1 per cent	20	c.c.
Potassium carbonate (anhydrous), 1 per cent	15	c.c.
Pyridine (pure)	1.6	c.c.

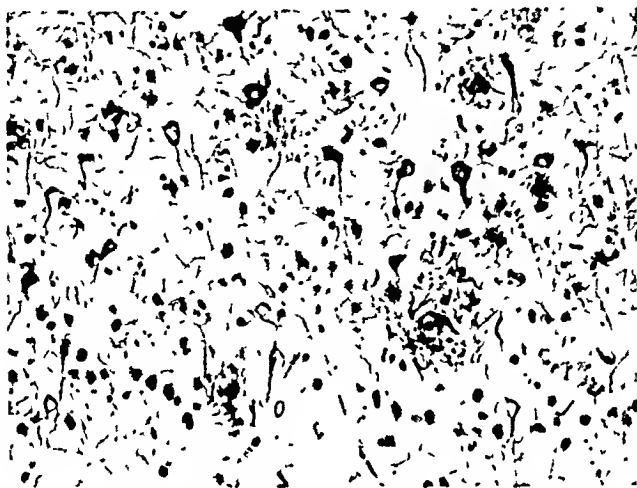


Fig. 1.—Alzheimer's neurofibrillary change in cerebral cortex. Pyridine silver stain (section 25μ), seven minutes $\times 200$.

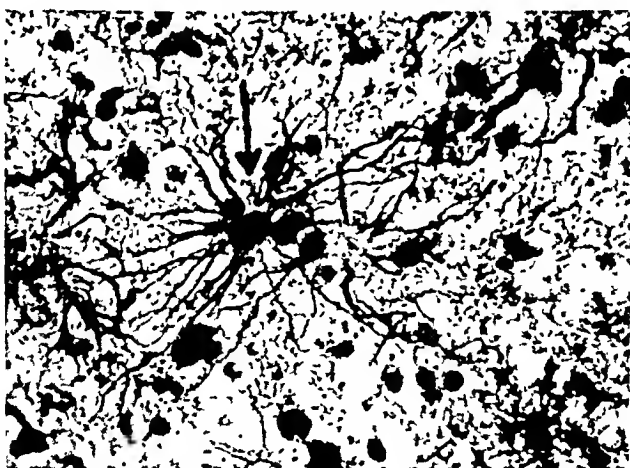


Fig. 2.—Fibrillary astrocytes. Focal level on central astrocyte (section 25μ) $\times 400$. Pyridine silver stain, nine minutes.

5. Transfer directly to excess of 10 per cent formalin (neutralized with calcium carbonate) for about one minute.

6. Dehydrate in alcohols and xylols in usual manner and mount in balsam. Reticular fibers stain black. Nuclei are not stained black by silver.

The silver pyridine solution is prepared fresh just before staining and may be used for one to three hours. Merek's and Eastman Kodak's chemically pure

pyridine have been found satisfactory. Toning with gold chloride and the application of counterstains may be employed in the manner described in texts on staining methods. For routine purposes this is not usually necessary.

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THE QUANTITATIVE KAHN TEST: RESULTS WITH 2.5 PER CENT AND 0.9 PER CENT SALT SOLUTION COMPARED WITH THE DEGREE OF REACTIONS OF THE STANDARD KAHN TEST

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SINCE 1940 the routine laboratory tests for syphilis in the Division of Laboratories of the Tennessee Department of Public Health have been the presumptive and standard Kahn tests for blood specimens and the Kolmer complement fixation test for spinal fluid. All tests are performed in exact accord with outlines of methods by author serologists.^{1, 2} Antigen for the Kolmer complement fixation test is purchased from commercial sources. Antigen for the Kahn standard test is purchased from the University Hospital, Ann Arbor, Michigan. Both tests have been included in annual national evaluation studies.

With the advent of rapid treatment centers in the state, the need became acute for quantitative tests to determine the serologic status of patients with syphilis before and after treatment. At first these quantitative tests were sent to Dr. Reuben L. Kahn at Ann Arbor. Later the Central Laboratory of the Division of Laboratories was able to start making quantitative Kahn tests following the method given by Kahn³ in Supplement 11, Venereal Disease Information. For a while duplicate specimens were sent by the treatment centers to Kahn's Laboratory and to the Central Laboratory. Titers on these duplicate specimens were quite different, with Kahn's Laboratory obtaining consistently higher titers than the Central Laboratory. Investigation revealed that Kahn's Laboratory was using 2.5 per cent salt solution in dilutions and in the performance of the test, while the Central Laboratory was using 0.9 per cent salt solution. The Central Laboratory changed to 2.5 per cent salt solution, using a 6:1 serum antigen ratio, and no further significant differences were noted in the titers obtained in the two laboratories. The Central Laboratory took over the examination of rapid treatment center specimens for quantitative tests in 1944, as a special procedure.

The numbers of specimens handled as special procedures for the treatment centers have increased steadily, and at present the Central Laboratory is making over 4,000 such quantitative tests each month. This increasing load, in addition to increases in routine serologic work in a serologic section of limited space and personnel, has been of considerable concern. To cut down to some extent on the work involved, thought was given to the elimination of the standard Kahn test as a preliminary to the quantitative Kahn test. The standard test seemed a very poor screen for the quantitative test on specimens from previously diagnosed patients with syphilis when the work involved in the standard test was considered in relation to the numbers of such specimens eliminated from the quantitative test. As all specimens for the quantitative Kahn test were from

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previously diagnosed untreated patients, or from previously diagnosed treated patients, it was decided that the standard Kahn test could be safely eliminated as a preliminary to the quantitative Kahn test if the first tube of the quantitative test contained undiluted serum with the usual dilutions of serum in the remaining tubes. When this was tried in practice, it was found that certain sera gave negative or doubtful reactions in the undiluted state but gave positive reactions in one or more of the dilutions. The standard Kahn test applied to these sera gave negative or doubtful reactions.

We have considered the standard Kahn test as quantitative in its differentiation of negative and weakly positive sera with the quantitative Kahn test in direct correlation and of value in determining the potency of strongly positive sera undifferentiated by the standard test. This conception is apparently in error when 2.5 per cent salt solution is used in the quantitative test, as it is not reasonable to consider the number of specimens we encountered that gave titers as due to prozones.

The fact that titers were obtained on negative or doubtful standard Kahn tests was disturbing, as patients are returned to rapid treatment centers one year after initial treatment if they show 10 or more Kahn reacting units (2 plus reaction in 1:2.5 dilution or higher). Before retreatment at medical centers, the serologic record of the individual is reviewed: if the serologic titer has leveled off at 10 units or more retreatment is given; if the record shows a consistent, continued falling titer the patient is re-evaluated at monthly intervals for two months; if further drops occur the patient is not retreated and is followed for additional periods.

The plan of eliminating the standard Kahn test as a preliminary to the quantitative Kahn test was abandoned as was the use of 2.5 per cent salt solution in the quantitative Kahn test. The quantitative Kahn test employing 2.5 per cent salt solution (6:1 serum antigen ratio) was applied experimentally to specimens received for routine diagnostic test. The specimens titrated were unselected other than that those chosen were specimens with sufficient serum giving normal types of reaction in the following groups: (1) negative standard Kahn; (2) doubtful standard Kahn; (3) 2 plus standard Kahn; (4) 3 plus standard Kahn; and (5) 4 plus standard Kahn. No specimens giving atypical or reverse reactions were included, as such abnormal reactions are usually given by highly potent sera which are likely to yield prozone reactions. Results obtained with the quantitative Kahn test, employing 2.5 per cent salt solution on these five groups of specimens, are given in Table I.

For comparative purposes the quantitative Kahn test employing 0.9 per cent salt solution (6:1 serum antigen ratio) was made on approximately the same numbers of routine specimens giving normal types of 3 plus and 4 plus reactions. No specimens giving atypical or reverse reactions were included. Results obtained with the quantitative Kahn test employing 0.9 per cent saline on these two groups of specimens are given in Table II.

DISCUSSION

It is understood that the quantitative Kahn test is intended only for use with sera giving strongly positive reactions with the standard Kahn test. How-

TABLE I

STANDARD KAHN TEST RESULTS	TOTAL SPECI- MENS	NEGATIVE IN ALL DILUTIONS 0, 1, 2, 3, OR 4 KAHN UNITS BASED ON STANDARD TEST	QUANTITATIVE KAHN TEST—2.5 % SALT SOLUTION (6:1 SERUM ANTIGEN RATIO)											
			POSITIVE 1:2.5 DILUTION KAHN UNITS	POSITIVE 1:5 DILUTION KAHN UNITS	POSITIVE 1:10 DILUTION KAHN UNITS	POSITIVE 1:20 DILUTION KAHN UNITS	POSITIVE 1:40 DILUTION KAHN UNITS	POSITIVE 1:80 DILUTION KAHN UNITS	POSITIVE 1:100 DILUTION KAHN UNITS	POSITIVE 1:150 DILUTION KAHN UNITS	POSITIVE 1:200+ DILUTION KAHN UNITS			
			55	30	27	10	18	1	0	0	0			
Negative (-)	745	604	55	30	27	10	18	1	0	0	0			
Doubtful														
(± and 1+)	690	165	212	196	85	25	4	0	0	1	2			
Positive (2+)	551	57	120	186	137	39	11	0	0	0	1			
Positive (3+)	527	33	80	195	165	46	7	0	0	0	1			
Positive (4+)	1035	4	10	68	280	320	308	11	11	6	14			

TABLE II

STANDARD KAHN TEST RESULTS	TOTAL SPECI- MENS	NEGATIVE IN ALL DILUTIONS 3 OR 4 KAHN UNITS BASED ON STANDARD TEST	QUANTITATIVE KAHN TEST—0.9 % SALT SOLUTION (6:1 SERUM ANTIGEN RATIO)											
			POSITIVE 1:2.5 DILUTION KAHN UNITS	POSITIVE 1:5 DILUTION KAHN UNITS	POSITIVE 1:10 DILUTION KAHN UNITS	POSITIVE 1:20 DILUTION KAHN UNITS	POSITIVE 1:40 DILUTION KAHN UNITS	POSITIVE 1:80 DILUTION KAHN UNITS	POSITIVE 1:100 DILUTION KAHN UNITS	POSITIVE 1:150 DILUTION KAHN UNITS	POSITIVE 1:200+ DILUTION KAHN UNITS			
			8	0	0	0	0	0	0	0	0			
Positive (3+)	553	545	284	227	150	62	19	2	1	0	0			
Positive (4+)	1041	295	284	227	150	62	19	2	1	1	0			

ever, it is felt that this restriction has been imposed because it was considered that sera giving negative or weakly positive reactions with the standard Kahn test would invariably give negative results with the quantitative Kahn test and not because it was thought that confusion would result from the titration of such specimens.

When the quantitative Kahn test employing 2.5 per cent saline (Table I) was applied to sera giving negative standard Kahn reactions, 19 per cent of these specimens gave a titer of 10 or more Kahn reacting units. Titers of 10 or more Kahn reacting units were obtained with 76 per cent of specimens giving doubtful standard Kahn reactions. Titers of 10 or more Kahn units were obtained with 89 per cent of 2 plus specimens, 93 per cent of 3 plus specimens, and over 99 per cent of 4 plus specimens.

When the quantitative Kahn test employing 0.9 per cent saline (Table II) was applied to approximately the same numbers of 3 plus and 4 plus specimens, it was found that less than 2 per cent of 3 plus specimens and only 71 per cent of 4 plus specimens gave titers of 10 or more Kahn units.

It seems logical to expect that a certain number of specimens giving a 4 plus reaction are only potent enough to produce this 4 plus reaction. The value of the quantitative test is in the differentiating or grading of these 4 plus or strongly positive specimens into those that are 4 plus, 40 plus, or 400 plus.

CONCLUSIONS

The results of this study indicate that the quantitative Kahn test employing 2.5 per cent salt solution is on a different sensitivity level than the standard Kahn test. There appears to be little correlation between the degree of reaction obtained with the standard Kahn test and the titer obtained with the quantitative Kahn test employing 2.5 per cent salt solution. The standard Kahn test and the quantitative Kahn test employing 0.9 per cent salt solution (6:1 serum antigen ratio) seem to be on the same sensitivity level. There appears to be a direct relation between the reaction obtained with the standard Kahn test and the titer obtained with the quantitative Kahn test employing 0.9 per cent salt solution. From our observations it seems that the exaggeration of the potency of syphilitic serum through the use of an overly sensitive quantitative test is of doubtful value and can be justified with difficulty where patients are re-treated on the basis of an arbitrary low titer after one year.

SUMMARY

Titers obtained in quantitative Kahn tests employing 2.5 per cent and 0.9 per cent salt solution are each compared with results obtained in the standard Kahn test on sera giving normal typical reactions. Tables are presented showing differences.

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A NOTE ON QUANTITATIVE KAHN TESTS EMPLOYING 0.9 AND 2.5 PER CENT SALT SOLUTION SYSTEMS

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INQUIRIES are occasionally received in this laboratory regarding the technique of the quantitative Kahn² test.* This test is not performed identically by different workers. Some employ serial dilutions of serum with 0.9 per cent NaCl solution and others employ serial dilutions of serum with 2.5 per cent NaCl solution. The quantitative test with 2.5 per cent salt solution is generally more sensitive than that with 0.9 per cent salt solution to the extent of approximately one or two additional serum dilutions. Thus, a serum that gives an end point in a 1:20 dilution with 0.9 per cent salt solution may give an end point in 1:30 or 1:40 dilution with 2.5 per cent salt solution.

In the reading of the results of the quantitative test with 2.5 per cent salt solution, emphasis has been given to the fact that the negative reactions tend to show clouding and precipitation on standing. This occurs even when they stand for relatively short periods, such as five to ten minutes. For this reason the results should be read without delay after the addition of the 0.5 c.c. amounts of 2.5 per cent salt solution, following the three-minute shaking period. Apparently it is this concentration of salt solution which causes the clouding and precipitation on standing. Shaking by hand for a few seconds disperses such precipitates and renders the tests clear again. Those workers who have not paid strict attention to this shaking of the tubes just before reading soon found themselves reading particles due to standing of the tests in the presence of the 2.5 per cent salt solution.

Some years ago we recommended the quantitative technique with the 2.5 per cent salt solution, because studies in this laboratory indicated that the salt concentration favors syphilitic reactions and renders them more sensitive than 0.9 per cent salt solution. The increased sensitivity seemed desirable because the quantitative test was then used largely in known cases of syphilis in the follow-up of therapy.

There are, however, points in favor of the use of quantitative Kahn tests with 0.9 per cent salt solution. During recent years quantitative tests have been used as an aid in diagnosis; hence, the high sensitivity of the 2.5 per cent system may also introduce an element of nonspecificity in certain cases. The tests with the 0.9 per cent system do not show a tendency toward clouding on standing and, therefore, do not require agitation immediately before reading. Furthermore, quantitative results with the 0.9 per cent system show closer correlation with the results of the standard Kahn test than quantitative results

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*Kahn, R. L.: Technique of Standard Kahn Test and of Special Kahn Procedures, Ann Arbor, 1946, University of Michigan Press, p. 22.

with the 2.5 per cent system. Thus, in order to avoid confusion between the 1 to 4 plus terminology of the standard Kahn test and the unit terminology of the quantitative test, we arbitrarily correlated the two terminologies as follows: One plus, 2 plus, and 3 plus in the standard test were arbitrarily considered as 1 unit, 2 units, and 3 units in the quantitative test. Sera giving 1, 2, or 3 plus reactions with the standard Kahn test generally give negative reactions on serial dilution of serum. Occasionally it will be found that a serum giving a 2 plus reaction, and particularly a 3 plus reaction in the standard test, will give a titer of 10 or 20 or higher in the quantitative test. The results are then reported to physicians with the explanation of this exceptional behavior of the serum. However, when performing quantitative tests with the 2.5 per cent NaCl system with weakly positive sera, greater numbers will be found to give quantitative titers. Hence, 1, 2, or 3 plus reactions with the standard test cannot, strictly speaking, be considered as equivalent to 1, 2, or 3 units in the quantitative test.

In this laboratory quantitative tests with both the 0.9 per cent and 2.5 per cent NaCl systems are employed in most cases. This scheme is obviously not feasible for general application. As to the choice between the two quantitative techniques, we believe that in view of the fact that quantitative Kahns are no longer used almost exclusively as serologic checks on therapy in syphilis, but are used increasingly as an aid in the establishment of a diagnosis, the 0.9 per cent NaCl system should be preferred.

A SIMPLE AND SAFE METHOD FOR THE GASTRIC INSTILLATION OF FLUIDS IN THE RAT

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THE rat has been used as an experimental animal in the field of nutrition for many years. More recently it has gained an important place for the study of problems in digestion. Both of these fields of application have made it most desirable that a rapid and safe method for introducing measured amounts of fluid into the stomach be devised.

As early as 1908 Marks¹ employed a silk-rubber catheter for the stomach tube feeding of mice. His method required two operators. In 1943 Ferrill² described the use of a metal cannula made from an injection needle carrying a small perforated brass knob. He claims safety and speed for the method. Methods utilizing a rubber catheter always involve the problem of preventing the rat from biting the tube. Most operators^{3, 4} employed a wooden mouth gag with a central hole for the passage of the catheter. We have tried this method and found it cumbersome and time consuming. The wooden mouth gag often hurts the animal, causes it to struggle, and not infrequently injures the mouth of the animal. We found the suspension method described by Machella and Griffith⁴ too time consuming to be used for large groups of animals. We have had no experience with the procedure recently reported by Lehr,⁵ but from its description it involves many more steps than the very simple and safe method we have evolved in our laboratory. After a little experience the loss of animals from intubation by this method is all but eliminated.

METHOD

A No. 8 French rubber catheter with a depressed eye is used as the stomach tube. With the rat resting on the table and its head facing toward the operator's right, the left hand of the operator is spread across the animal; the thumb and index finger are so placed that the middle of the distal phalanx of the former is at the angle of the mouth, while the latter straddles the base of the skull of the animal (Fig. 1). This arrangement gives these two fingers control of the head. The palm of the left hand fixes the animal to the table by gentle pressure on its back. In this way all four feet of the animal are immobilized. The thumb is then slid upward and backward, a procedure which draws the tissues back, opens the jaws, and at the same time slightly turns the head of the animal toward the operator (Fig. 2). This position must be maintained until the end of the procedure. The tip of the rubber catheter is moistened with normal saline (not oil) and is inserted over the tongue and

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passed either along the right or the left side of the mouth, keeping the tube as close to the cheek as possible. By this maneuver the tube moves readily down the esophagus into the stomach (Fig. 3). If any resistance to the passage of



Fig. 1.



Fig. 2.

the tube is met, the tube should be removed and reintroduced. The length of catheter necessary to intubate the stomach depends upon the size of the animal. With a little experience, impingement of the tip of the tube upon the

stomach wall is readily recognized. When measured amounts of fluids are to be injected, the catheter is attached to a Luer syringe and the fluid is drawn through the catheter before being introduced into the stomach. This permits one to ignore the fluid which remains in the catheter at the end of the injection. After the fluid is slowly injected into the stomach, the catheter, with syringe attached, is quickly withdrawn.



Fig. 3.

We have intubated several thousand rats by this method. In some the catheter was inserted three times a day for several months without apparently causing any discomfort to the animal. The technique may be learned quickly, and after a little experience one operator can handle sixty animals an hour. We have used the method successfully on animals weighing as little as 60 grams each,

SUMMARY

A simple and safe method for gastric intubation and for the gastric instillation of fluids in the rat is described.

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BOOK REVIEWS

Human Embryology. By *Bradley M. Patten*, Ph.D., Professor of Anatomy, University of Michigan School, Ann Arbor, Michigan. The Blakiston Company, Philadelphia, 1946. Price \$7.00. Cloth with 776 pages (illustrated).

The recent publication of the new textbook of *Human Embryology* by Professor B. M. Patten is an unusually serviceable and timely contribution to the study of the subject. For a textbook covering material so full of detail, little of importance has been omitted. The volume is admirable for the clarity with which it is written and is highly recommended. Because the book is scholarly and gives a somewhat dogmatic approach to the anatomy of the developing human embryo and fetus, it will probably be adopted as a standard textbook in many medical schools. The author does not stray from his purpose of designing the work primarily for students of medicine and for physicians. Embryology is presented in a practical manner to both student and teacher, on paper that is relatively good for these times. The compilation of subject matter is amazingly complete and accurate, and the writer includes few text references to disrupt the continuity of the discourse. However, a list of 939 articles is arranged according to subject matter in the bibliography. The index is both complete and easy to use.

While the aim of the book is both analytical and informative, it presupposes a general knowledge of the subject, particularly that of the development of the chick. Emphasis is given to the comprehension of developmental processes rather than to illustration of any particular form, other than that of man. Comparative anatomical aspects of the subject, therefore, are minimal. It is apparent, however, that the author is well aware that many features of human embryonic development are incomprehensible unless viewed in the light of development of some lower forms. The section on the phylogenetic development of muscles of facial expression, for example, is certainly adequate. The author's keen insight into the subject, appreciation of students' difficulties, and broad experience in interpreting our present knowledge of human developmental processes is apparent in the topics selected for detailed discussion.

Organization of subject matter is such that it provides a direct continuity between consecutive subjects, making for a unified discourse on human development rather than a series of individual treatises. Following the newer trend, proper names have been replaced largely by names descriptive of the structure; the term *ovum* is used only in the restricted sense. Beginning with a short account of the history of embryology and the purpose of embryology in the medical curriculum, the text passes to a logical consideration of male and female reproductive organs. The sexual cycle is discussed adequately in the chapter devoted to processes of fertilization and sex determination. Segmentation of the zygote and establishment of the germ layers and the early embryonic body are well described, especially the section on formation of mesoderm and the origin of the primitive streak. The Hertig-Rock 11- and 12-day-old human embryos are discussed, but the more recent 7- and 9-day-old human embryos are not included. Chapter five gives a good insight into the early differentiation of various regions of the body from primitive streak material. It is especially serviceable as a guide to the study of the 5 to 15 mm. pig embryo and is a good substitute for a separate section devoted to laboratory instructions. Of value also is a concise but pertinent account of early differentiation of the organ systems in very young human embryos which facilitates the comprehension of a complete picture of embryos, as a whole, up to six weeks of age, an age comparing favorably with that of 5 to 15 mm. pig embryos. Subsequent stages of growth of each organ system are considered in detail in later chapters. The sections on fetal membranes and the placenta, as well as those concerned with the organ systems, are well balanced and provide a good

embryologic foundation for advanced medical study. The chapter on age and body growth and form includes a number of authoritative graphs. Those depicting the growth of the body in stature during prenatal and postnatal life and mean weights of organs at different periods of fetal growth will be very useful. The description of twins, double monsters, and teratology leaves little to be desired. Causative factors operative in the production of developmental anomalies are also discussed. Particular stress is placed throughout the text on the more common developmental anomalies in human embryos. These are well described, admirably illustrated, and of value to clinicians. The section on Spina bifida is an excellent example, along with others cited, of the author's belief that it is difficult to try to explain all anomalies on the basis of the theory of developmental arrests.

Advanced stages of organogenesis, as well as histogenesis and frequent recourse to pictorial and descriptive accounts of postnatal development and comparisons between the newborn and adult, permeate the text. This highly desirable practice facilitates the correlation of embryology with the study of histology and gross anatomy. Functional aspects of the work are intimately associated with developing structures throughout the greater part of the book. Outstanding sections of the text are those dedicated to the connective tissues and skeleton, nervous system, body cavities and mesenteries, urogenital system, and postnatal changes in circulation. Emphasis is also ascribed to histogenesis of the cerebral cortex, retina, general sense organs, digestive system, endocrine organs, and gonads. Much of the illustrative data has been obtained from the University of Michigan slide collections. The numerous illustrations are well chosen and instructive. While they have been designed for more than sheer beauty, it would be difficult to surpass many of these illustrations in artistic merit. Illustrated gross dissections tell an accurate story and serve as excellent teaching guides. The figures showing postnatal development of the skeletal, muscular, nervous, digestive, and circulatory systems should be of great value to those less expert than the author.

The author clearly portrays embryology as a basis for understanding other anatomical sciences. He points out that it will lead the student "beyond anatomical memorizing to a comprehension of anatomy." It will be recognized that such a textbook, despite the advances of experimental embryology, must contain some degree of dogmatism. The author's experience and contributions, however, have made his opinions well worth knowing and worthy also of the dignified format chosen.

J. H. VAN DYKE.

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